Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Molecular analysis of virulence mechanisms associated with Adherent-Invasive *Escherichia coli* (AIEC)

Adam O'Driscoll

A thesis submitted for the degree of Doctor of Philosophy

National University of Ireland, Cork

Department of Microbiology

October 2012

Head of Department: Prof Gerald F. Fitzgerald

Supervisor: Dr David Clarke

Contents

Declaration

I declare that this thesis has not been previously submitted as an exercise for a degree, either at University College Cork or elsewhere. Furthermore, I declare that this thesis is my own work and where other sources of information have been used, they have been acknowledged.

Adam O Driscoll

10 October 2012

Acknowledgements

Firstly I would like to take this opportunity to thank my supervisor Dr David Clarke for his help, encouragement and enthusiasm for the project throughout my PhD. His door was always open and his advice, endurance and interest over the years have been invaluable. Thank you!! I am deeply indebted to all those from lab 405, past and present, for their advice and assistance. Very special thanks to Catherine, Lea, Aoife, Marc, Elaine, Eoghan, John, Ian and Emma!! Thank you for making my time in UCC an enjoyable one.

Thanks also to the staff of the Microbiology department and APC, in particular Liam Burgees and Margaret Higgins. I would also like to acknowledge the help of Dr. Susan Joyce who provided suggestions and ideas and also taught me the techniques required to complete the amoeba experiments. A special thanks to Dr Jennifer Mahony for sharing her knowledge and experience and for introducing me to the world of research during my undergraduate!

Thank you to my parents, Sean and Patsi who have provided endless support and encouragement. I will forever be grateful for their immeasurable assistance. Thank you to my brothers and sister, Roy, Mark, Jason, Jonathan and Tara, the most incredible people I have ever met and I'm lucky enough to call them my family!!

Thank you also goes to Seamus and Joan for being so welcoming!! Finally, thank you to Sheila who has been there from the start and experienced every minute with me (including listening to my never ending ranting)! Her love, support and smile have kept me going. There's nobody out there like her and I couldn't have done it without her, Thank you!!

List of abbreviations

^{o}C	Degrees celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
AIEC	Adherent invasive *Escherichia coli*
Amp	Ampicillin
APC	Antigen presenting cells
ASKA	A Complete Set of *E. coli* K-12 ORF Archive
ATP	Adenosine triphosphate
bp	Base pairs
BER	Base-excision repair
BFP	Bundle-forming pilus
CD	Crohn's disease
cfu(s)	Colony forming units(s)
Cm	Chloramphenicol
DAF	Decay-accelerating factor
DC (s)	Dendritic cell (s)
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleutide 5'-triphosphate
EAF	EPEC adherence factor
EDTA	Ethylenediaminetetraacetic acid
FAE	Follicle associated epithelium
Fig.	Figure
FLP	Flippase recombination enzyme
FRT	Flippase recognition target
G	Grazing phenotype
g	Gram(me)
gfp (*gfp*)	Green fluorescent protein (gene)
GI	Gastrointestinal
Gm	Gentamicin
GR	Grazing resistance

GWAS	Genome-wide association studies
dH_2O	Distilled water
H_2O_2	Hydrogen peroxide
HK	Histidine kinase
HOCL	Hypochlorous acid
HPt	Histidine-containing phosphotransfer domain
IBD	Inflammatory bowel disease
IL1-β	Interleukin 1 beta
IL23-R	Interleukin 23 Receptor
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-b-D-thiogalactoside
IRGM	Immunity-related GTPase M
Km	Kanamycin
kb	Kilobase
LB	Luria-Bertani media
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
M	Molar
MES	2-(N-morpholino)ethanesulfonic acid
MIC	Minimum inhibitory concentration
MDP	Muramyl dipeptide, N-acetylmuramyl-L-alanyl-D-isoglutamine
mg	Milligram
ml	Millilitre
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mM	Millimolar
MMR	Mismatch repair
MOI	Multiplicity of infection
MPO	Myeloperoxidase
mV	Millivolts
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NCBI	National Centre for Biotechnology Information
NER	Nucleotide excision repair

NF_KB	Nuclear factor *kappa*-light-chain-enhancer of activated *B* cells
NO^-	Nitric oxide
O_2^-	Superoxide anion
OCTN	Organic cation transporter
OD	Optical density
OMP	Outer membrane protein
OMV (s)	Outer membrane vesicle (s)
$ONOO^-$	Peroxynitrite
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
pmol	picomole
PP	Peyer's patches
PRR	Pattern recognition receptor
rcf	Relative centrifugal force (*g)*
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Response regulator
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
sp	Species
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TCS	Two component system
TEM	Transmission electron microscopy
Tn	Transposon
TNF-α	Tumour necrosis factor alpha
UC	Ulcerative colitis
UTI (s)	Urinary tract infection (s)
UV	Ultra violet

v/v	Volume/volume
w/v	Weight/volume

Abstract

Crohn's Disease (CD) is a chronic inflammatory bowel disease of unknown etiology. Recent work has shown that a new pathotype of *Escherichia coli*, Adherent Invasive *E. coli* (AIEC) may be associated with CD. AIEC has been shown to adhere to and invade epithelial cells and to replicate within macrophages (together this is called the AIEC phenotype). In this thesis, the AIEC phenotype of 84 *E. coli* strains were determined in order to identify the prevalence of this phenotype within the *E. coli* genus. This study showed that a significant proportion of *E. coli* strains (approx. 5%) are capable of adhering to and invading epithelial cells and undergoing intramacrophage replication. Moreover, the results presented in this study indicate a correlation between survival in macrophage and resistance to grazing by amoeba supporting the coincidental evolution hypothesis that resistance to amoebae could be a driving force in the evolution of pathogenicity in some bacteria, such as AIEC.

In addition, this study has identified an important regulatory role for the CpxA/R two component system (TCS) in the invasive abilities of AIEC HM605, a colonic mucosa-associated CD isolate. A mutation in *cpxR* was shown to be defective in the invasion of epithelial cells and this defect was shown to be independent of motility or the expression of Type 1 fimbriae, factors that have been shown to be involved in the invasion of another strain of AIEC, isolated from a patient with ileal CD, called LF82. The CpxA/R TCS responds to disturbances in the cell envelope and has been implicated in the virulence of a number of Gram negative pathogens. In this study it is shown that the CpxA/R TCS regulates the expression of a potentially novel invasin called SinH. SinH is found in a number of invasive strains of *E. coli* and *Salmonella*. Moreover work presented here shows that a critical mechanism underpinning AIEC persistence in macrophages is the repair of DNA bases damaged by macrophage oxidants.

Together these findings provide evidence to suggest that AIEC are a diverse group of *E. coli* and possess diverse molecular mechanisms and virulence factors that contribute to the AIEC phenotype. In addition, AIEC may have gone through different evolutionary history's acquiring various molecular mechanisms ultimately culminating in the AIEC phenotype. The gastrointestinal (GI) tract harbors a diverse

microbiota; most are symbiotic or commensal however some bacteria have the potential to cause disease (pathobiont). The work presented here provides evidence to support the model that AIEC are pathobionts. AIEC strains can be carried as commensals in healthy guts however, when the intestinal homeostasis is disrupted, such as in the compromised gut of CD patients, AIEC may behave as opportunistic pathogens and cause and/or contribute to disease by driving intestinal inflammation.

Chapter 1.0: Introduction

1.1 Introduction to Crohn's Disease

Crohn's Disease (CD) and Ulcerative colitis (UC) are the two major pathologies of inflammatory bowel disease (IBD). Chronic disorders of the gastrointestinal tract, including CD and UC, have a combined prevalence of approximately 150-200 cases per 100,000 individuals in the Western World (Loftus Jr., 2004) with CD alone affecting approximately 500,000 people in both North America and Europe (Naser *et al.*, 2012). The prevalence of CD among males and females shows little to no difference with symptoms typically developing in late adolescence and early adulthood, although symptoms have been shown to develop at all ages (Loftus Jr., 2004). UC only affects the mucous membrane of the colon whereas CD can affect any portion of the gastrointestinal (GI) tract from mouth to anus but is most common in the ileum. CD is characterised by chronic granulomatous inflammation of the intestine resulting in edema (swelling), increased blood flow and ulcerations (Naser *et al.*, 2012). Such ulcerations can penetrate the bowel wall and patients often suffer from bloody diarrhoea, weight loss and chronic abdominal pain (Barnich, 2003). Moreover, signs and symptoms may occur in areas independent of the GI tract for example as joint pain, skin lesions, inflammation of the eyes (Baysoy *et al.*, 2011; Ceresara *et al.*, 2010; Weiser *et al.*, 2011) and recently multinodular mass lesions on kidneys (Semjen *et al.*, 2011).

CD, originally described by Dr. Burrill B. Crohn and his colleagues in the 1930s (Crohn *et al.*, 1984; Crohn. B.B *et al.*, 1932), is a heterogeneous entity and is separated into 3 phenotypes or disease behaviours: non-stricturing and non-penetrating, stricturing and, finally, penetrating CD (Brand *et al.*, 2005; Louis *et al.*, 2003; Naser *et al.*, 2012). Strictures develop in areas of chronic inflammation where scarring occurs narrowing the width of the lumen (Spinelli *et al.*, 2009). Interestingly, the behaviour of CD has been shown to change over the course of the disease. In most cases patients present with non-stricturing non-penetrating disease at diagnosis while after 25 years the majority harbour either a structuring or penetrating pattern (Louis *et al.*, 2003). Currently, there is no cure for CD and as a result treatment involves supressing the immune system using medication including

Infliximab, 5-aminosalicylates and steroids to relieve the symptoms. Over 80% of CD patients will undergo surgery and approximately one-third of those patients require reoperation (Heimann *et al.*, 1998; Lee *et al.*, 2012)

The etiology of CD is still unknown; however, it has become widely accepted that CD develops due to an abnormal inflammatory response to commensal enteric bacteria providing continuous antigenic stimulation and activation of the intestinal mucosal immune system leading to intestinal injury in genetically susceptible hosts, thus characterising this condition as an autoimmune disease (see fig 1.1) (Eckburg & Relman, 2007; Strober *et al.*, 2007; Xavier & Podolsky, 2007). Moreover, siblings of individuals affected by CD have a higher risk of acquiring the disease and recent studies have identified specific genetic variations/polymorphisms contributing directly to CD (Libioulle *et al.*, 2007; Naser *et al.*, 2012).

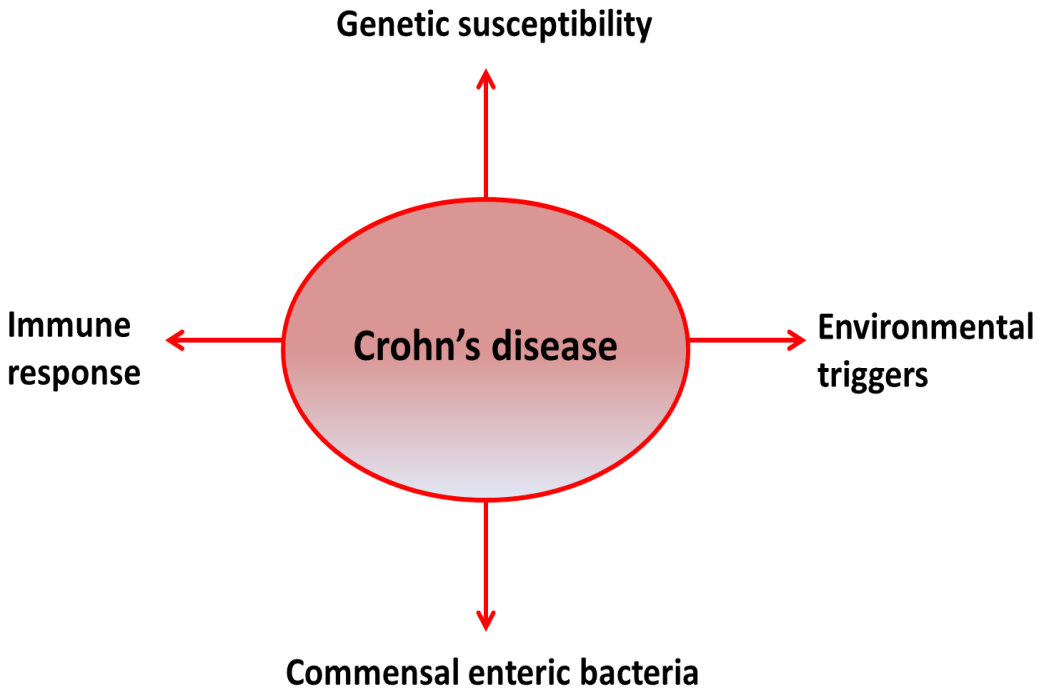


Figure 1.1 Pathogenesis of Crohn's disease: The model

Interaction of a number of factors can contribute to the chronic intestinal inflammation in a genetically susceptible host (Modified from Sartor, R.B 2006).

1.1.1 Gene polymorphisms associated with Crohn's Disease

In recent years, genome-wide association studies (GWAS) using high-throughput single nucleotide polymorphism (SNP) genotyping technologies have been applied to robustly associate specific variants with many complex diseases such as cancer and diabetes (Grant *et al.*, 2008; Hampe *et al.*, 2007). Such studies have established that many genetic loci contribute to inflammatory bowel disease, especially in CD (see Table 1.0). The most consistently and strongly associated variants have been in the autophagy-related 16-like 1 (*ATG16L1*), *NOD2/CARD15* and the interleukin 23 receptor (*IL23R*) genes (Cho, 2008; Csongei *et al.*, 2010). It is important to note that not everyone who has these polymorphisms develops CD, therefore other etiologic factors must underlie CD pathogenesis.

Table 1.0 Gene polymorphisms in Crohn's Disease (modified from Naser *et al* 2011)

Genes	Chromosome	Genetic variation	Function	Ref
NOD2/CARD15	16q12-13	3020insC (1007fs) R702W G908R	Cytosolic pattern recognition receptor	(Cuthbert *et al.*, 2002; Hugot *et al.*, 2001; Lesage *et al.*, 2002; Ogura *et al.*, 2001a)
IBD5	5q31	SLC22A4(OCTN1) SLC22A5(OCTN2)	A 250 kb haplotype on chromosome 5	(Daly *et al.*, 2001; Peltekova *et al.*, 2004; Rioux *et al.*, 2001)
ATG16L	2q37.1	rs2241880	Encodes protein that forms part of a large protein complex necessary for autophagy. Ileal form of CD	(Grant *et al.*, 2008; Hampe *et al.*, 2007; Naser *et al.*, 2012; Prescott *et al.*, 2007; Rioux *et al.*, 2007)
IRGM	5q33.1	rs13361189 rs4958847	Involved in autophagy	(Palomino-Morales *et al.*, 2009; Parkes *et al.*, 2007)

IL23R	1q31	R381Q (rs11209026)	Regulates pro-inflammatory activities and the differentiation of Th-17 lymphocytes	(Di Meglio *et al.*, 2011; Duerr *et al.*, 2006; Pidasheva *et al.*, 2011)
TNFSF15	9q32v	7 SNPs identified within a 280 kb region on chromosome 9q32	Encodes a protein which is a cytokine belonging to the TNF ligand. Strongly associated with CD in Japanese patients, not European patients.	(Yamazaki *et al.*, 2005)

1.1.2 Crohn's Disease and Autophagy

Macroautophagy, referred to here as autophagy, is a catabolic intracellular degradation system in which cytoplasmic components are sequestered within double-membrane vesicles resulting in the formation of autophagosomes. These autophagosomes are then delivered to the lysosome for degradation by lysosomal hydrolases (Saito *et al.*, 2012). Cells use this pathway during nutrient starvation to break down and recycle non-vital cellular components and use them as nutrients (Naser *et al.*, 2012). Basal levels of autophagy occur in most cells as a homeostatic function allowing the turnover of cellular components and proteins. Autophagy is initiated when structural remodelling is required to remove damaged cytoplasmic organelles, for example during intracellular infection, accumulation of misfolded proteins, oxidative stress and nutrient starvation (see fig. 1.2) (Fritz *et al.*, 2011).

Autophagy plays a role in both innate and adaptive immunity by helping rid the cell of an invading pathogen due to the autophagosomes capacity to sequester and digest microorganisms (Naser *et al.*, 2012; Plantinga *et al.*, 2012). Autophagy plays a crucial role in loading antigens onto MHC class II molecules of antigen presenting cells (APCs) to induce adaptive immunity by coupling to the T-cell receptor of CD4+ T-cells (Plantinga *et al.*, 2012). Moreover, during the initiation phase of autophagy, Immunity-related GTPase M (IRGM) localises to bacteria-containing autophagic vacuoles (Fritz *et al.*, 2011). IRGM and autophagy have been implicated

in the clearing of intracellular organisms such as *Mycobacterium tuberculosis*, *Helicobacter pylori* and *Salmonella enterica* serovar Typhimurium (Gutierrez *et al.*, 2004; Kuballa *et al.*, 2008; Singh *et al.*, 2006; Zavros & Rogler, 2012). Consequently, if the gene/genes for autophagy are mutated it may cause a shift in the normal gut flora. The cell would be unable to respond to foreign antigens resulting in programmed cell death and tissue damage contributing to a possible cause of CD (Naser *et al.*, 2012).

1.1.2.1 Autophagy-related 16-like 1 protein like complex (*ATG16L1)*

Recently, a polymorphism in the autophagy gene *ATG16L1* was identified as a possible factor for the development of CD (Hampe *et al.*, 2007; Kuballa *et al.*, 2008; Rioux *et al.*, 2007). The ATG16L1 protein can be found in the colon, intestinal epithelial cells, small intestine, leukocytes and the spleen (Fujita *et al.*, 2008). To date approximately 30 autophagy-related genes (*atg*) have been identified and at least 17 of these *atg* genes have been shown to be related to autophagosome formation (Fujita *et al.*, 2008). Two ubiquitin-like systems are required for formation of the autophagosome, the Atg12 system and the LC3 system (see fig 1.2) (Naser *et al.*, 2012). With regards the Atg12 system, Atg12-Atg5 associates with Atg16L1 forming a ~800kDa protein complex (referred to as the Atg16L1 complex). A fraction of the complex localizes to isolation membranes and the complex is released from the membrane just before or after autophagosome completion (Fujita *et al.*, 2008).

Multiple independent association studies have shown that the G allele of SNP rs2241880 confer a strong risk for CD. A genome-wide survey of 19,779 SNPs, showed a Threonine to Alanine (T-300-A) substitution at amino acid position 300 within the N terminus of ATG16L (Hampe *et al.*, 2007). This SNP was found to be highly associated with CD by using a haplotype and regression analysis (Grant *et al.*, 2008; Libioulle *et al.*, 2007; Naser *et al.*, 2012). The presence of the T-300-A polymorphism within the *ATG16L1* gene on chromosome 2q37.1 leads to the development of dysmorphic and dysfunctional Paneth cells in the intestine of CD patients and consequently altered protein expression patterns, defective bacterial handling and defective autophagy (Cadwell *et al.*, 2008; Zavros & Rogler, 2012). A

recent study showed that *ATG16L1* knockout mice induced elevated levels of the pro-inflammatory cytokines IL-1β and IL-18 in response to microbial stimuli and interestingly these mice are more susceptible to DSS-induced colitis (Saitoh *et al.*, 2008). Furthermore, two polymorphisms in the autophagy gene *IRGM* on chromosome 5q33.1 were also significantly associated with CD (Parkes *et al.*, 2007). These polymorphisms were shown, by means of microRNA miR-196, to affect IRGM protein expression and, as a consequence, the ability to execute autophagic processes is affected (Brest *et al.*, 2011; McCarroll *et al.*, 2008; Plantinga *et al.*, 2012). The associations of these autophagy genes with CD strongly support the hypothesis that abnormal immune responses to intracellular pathogens contribute to the pathogenesis of CD. Interestingly, a recent study showed that a deficiency in *ATG16L1* confers protection *in vivo* against acute and latent UPEC infections. This could suggest that a deficiency in this key autophagy gene can protect against infection and could provide an explanation for the prevalence of this mutation (Wang *et al.*, 2012).

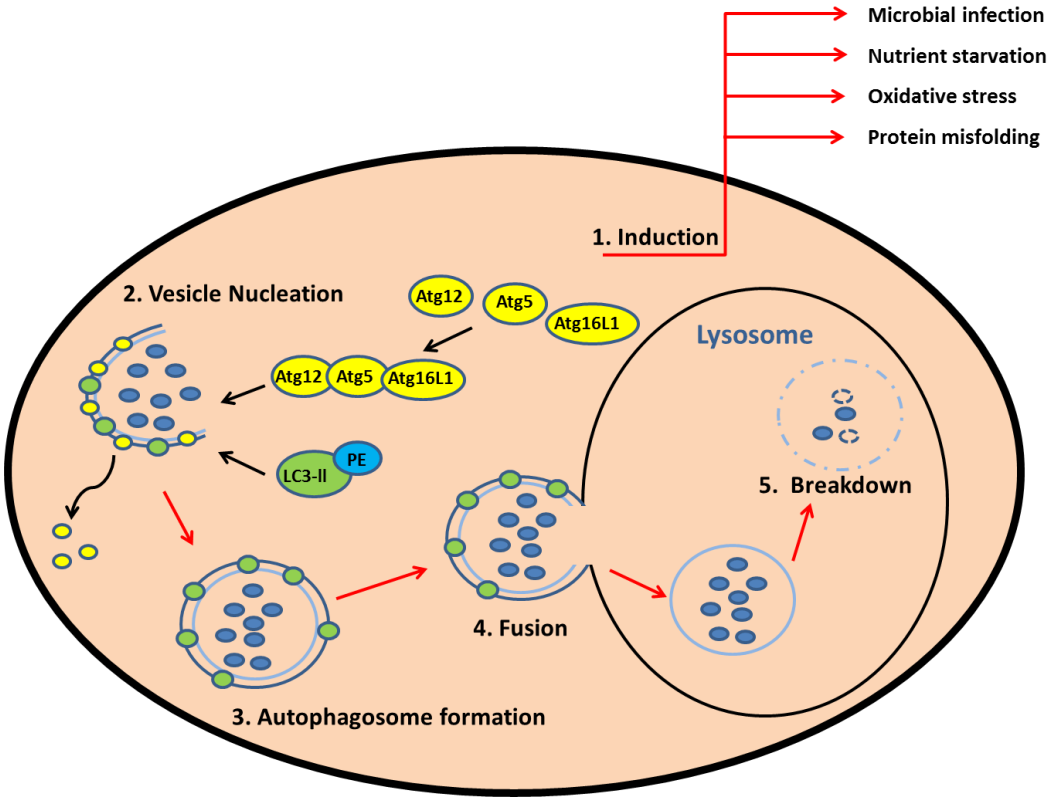


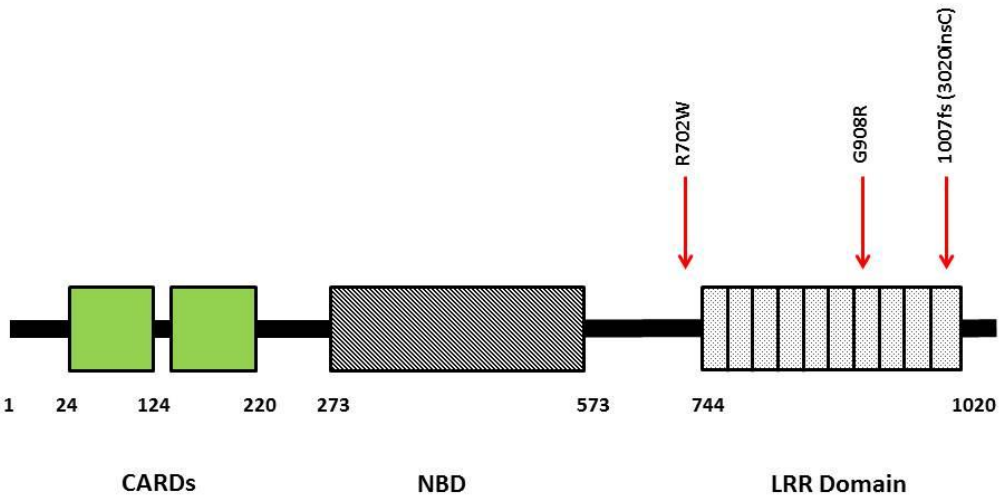
Figure 1.2: The processes inducing macroautophagy resulting in the degradation of cytoplasmic components. Following induction, a double membrane structure forms in the cytosol sequestering organelles, proteins etc. Atg12 and Atg5 fuse with Atg16 forming the Atg12-Atg5-Atg16L1 complex. This complex then localizes to the outer membrane of the forming autophagosome (steps 1-2). Upon autophagosome completion, the Atg12–Atg5–Atg16L1 complex recycles from the outer membrane, and only LC3 remains associated with the completed autophagosome. The autophagosome fuses with the lysosome and consequently the contents of the autophagosome are released into the lysosome for degradation by proteases (steps 3-5).

1.1.3 NOD2/CARD15

The Nucleotide-binding oligomerization domain containing 2 (NOD2)/Caspase Recruitment Domain Family member 15 (CARD15) has been characterized as the IBD1 locus on chromosome 16q12-13. This protein acts as a pattern recognition receptor (PRR) and plays an essential role in the immune system by regulating commensal bacterial flora in the intestine (Tsianos *et al.*, 2012). NOD2 encodes a tripartite protein consisting of two N-terminal caspase recruitment domains (CARDs) involved in apoptosis, a centrally-located nucleotide binding oligomerization domain (NBD) and a C-terminal domain of leucine-rich repeats (LRR) hypothesised to be involved in ligand recognition and protein-protein interactions (see Fig.1.3) (Hruz & Eckmann, 2011). Three main variants of the NOD2 protein, R702W, G908R and 1007fs have previously been shown to be strongly associated with CD. Interestingly, all 3 variants are located within, or close to, the C-terminal domain of LRR which interacts with bacterial products (see fig.1.3) (Cuthbert *et al.*, 2002; Lesage *et al.*, 2002). In a recent study, a large cohort of European patients with IBD showed that all three polymorphisms were strongly associated with CD and not UC. Moreover, the frequency of the *NOD2/CARD15* mutations was higher with CD and not UC. Interestingly, the frequency of the *NOD2/CARD15* mutations was higher in families affected with ileal-specific disease cases (Cuthbert *et al.*, 2002).

1.1.3.1 *NOD2/CARD15* function

NOD2/CARD15 belongs to a family of cytoplasmic proteins believed to be involved in the detection of bacteria by acting as an intracellular sensor for bacterial peptidoglycan (Torok *et al.*, 2009). Interestingly, the expression of NOD2/CARD15 was originally thought to be confined to cells of the monocyte lineage however expression is also found in intestinal epithelial cells of the small and large intestines, particularly in the specialised epithelial Paneth cells (Cuthbert *et al.*, 2002). The exact function of Paneth cells is unknown, but they are likely to contribute to host defence by secreting anti-bacterial substances in response to N-acetylmuramyl-L-alanyl-D-isoglutamine/muramyl dipeptide (MDP) found in peptidoglycan. The MDP is the minimal essential structure of bacterial cell wall peptidoglycan (PGN) and is conserved in both Gram-negative and positive bacteria (Lala *et al.*, 2003; Naser *et al.*, 2012).



CARDS: Caspase recruitment domains
NBD: Nucleotide binding domain
LRR Domain : Leucine-rich repeats

Fig 1.3 NOD2/CARD15 domain structure and the position of the polymorphisms associated with Crohn's disease.

The N-terminal portion contains two CARD domains known to play a role in apoptosis and NF-κB activation pathways. The NOD domain located in the middle portion of the protein is involved in protein self oligomerization and the C-terminal portion of the protein contains a leucine-rich repeat domain known to play a role in protein–protein interactions. Each of the three mutations occurs within or near the leucine-rich repeat and decrease the cells ability to activate NF-κB in response to peptidoglycan.

A *NOD2/CARD15* mutation is thought to diminish the function of Paneth cells thus reducing any antimicrobial activity and resulting in the development of ileal lesions. It has been reported that NOD2 triggering by MDP induces autophagy in antigen presenting cells such as dendritic cells (DCs) (Cooney *et al.*, 2009). NOD2-mediated autophagy was shown to be required for both bacterial handling and generation of major histocompatibility complex (MHC) class II antigen-specific $CD4^+$ T cell responses in DCs. Therefore DCs expressing CD-associated NOD2 polymorphisms are defective in autophagy induction, bacterial trafficking and antigen presentation.

NOD2/CARD15 is involved in the activation of nuclear factor-κB (NF-κB) (Iwanaga *et al.*, 2003; Ogura *et al.*, 2001b). The NOD2 protein combines with the serine threonine kinase receptor-interacting CLARP-associated kinase (RICK) to activate NF-κB (Kobayashi *et al.*, 2002). Although a cytosolic sensor, NOD2 is also localised to the plasma membrane and recruits RICK to activate NF-κB and IL-8 release (Barnich *et al.*, 2005; Lecine *et al.*, 2007). Compared to wild-type NOD2/CARD15, all of the three polymorphisms identified show a decreased ability to activate NF-κB in response to PGN (Bonen *et al.*, 2003). Moreover, CD patients with the frameshift mutation 1007fs, exhibit decreased defensin expression by Paneth cells. Defensins are cationic antimicrobial peptides that form pores in bacterial membranes (Ganz, 2003). As a result of the 1007fs mutation, NOD2 function is diminished allowing entry of bacterial populations into epithelial cells. This would be expected to result in an increase in bacterial persistence via impaired lysosomal destruction and immune mediated clearance (see fig. 1.4) (Cooney *et al.*, 2009) (Traub *et al.*, 2006; Van Limbergen *et al.*, 2007). This suggests that NOD2/CARD15 acts to protect the intestinal epithelium from bacterial invasion and that, in patients with CD, this protection mechanism function poorly allowing a shift in normal bacterial flora. Along with a number of factors such as environment, dysfunctional immune system and altered bacterial flora, this polymorphism can contribute to the pathogenesis of CD (Cuthbert *et al.*, 2002).

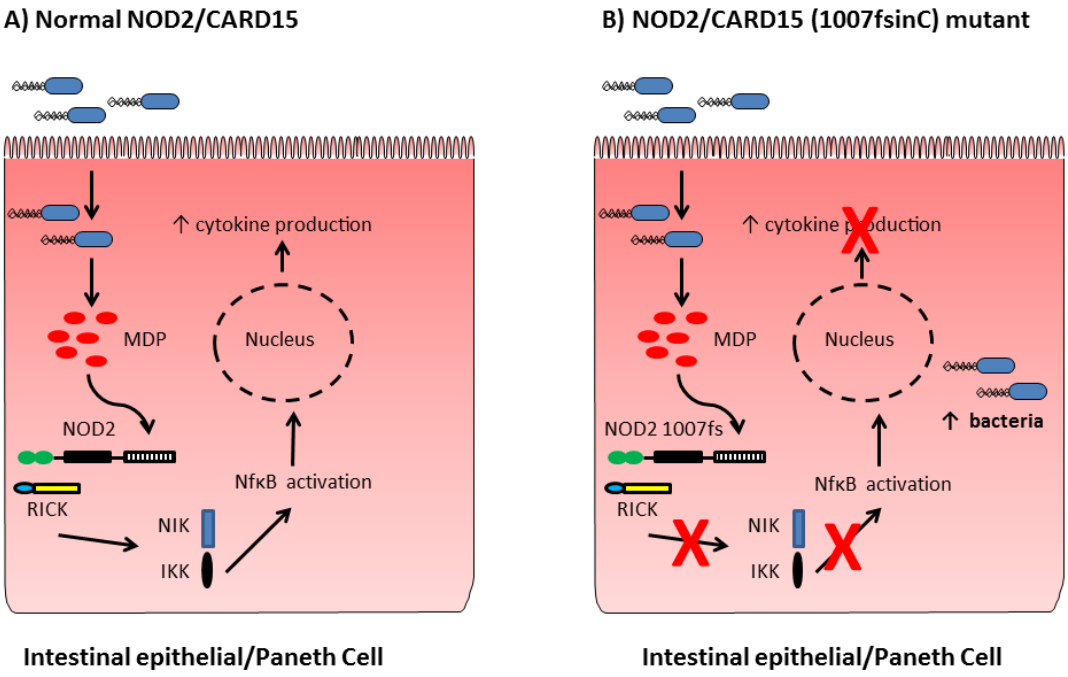


Figure 1.4: NOD2/CARD15 intracellular recognition of muramyl-dipeptide (MDP). A) During normal mucosal homeostasis, in response to MDP, a CARD in the NOD2 complex binds to the adaptor kinase RICK. Phosphorylation of IKK by RICK leads to downstream phosphorylation of the IKκ subunit of NFκB. Activated NFκB translocates into the nucleus and upregulates genes involved in cytokine production and defensin release. B) NOD2/CARD15 polymorphisms result in deficient epithelial cells responses which in turn lead to increased exposure to mucosal bacteria. The 1007fs variant results in deficient sensing of MDPs and diminished activation of NFκB resulting in decreased cytokine and defensin expression and increased intraepithelial bacteria.

1.1.4 Interleukin 23 Receptor (IL23-R)

It has been continuously reported that dysregulated pro-inflammatory cytokine production by mucosal macrophages and lymphocytes is implicated in the pathogenesis of CD (Boirivant *et al.*, 1999; Fuss *et al.*, 1996; Garcia de Tena *et al.*, 2006; Tsianos *et al.*, 2012). IL23-R is highly involved in the regulation of pro-inflammatory activities and the differentiation of T helper type (Th-17) lymphocytes which are critical for host defense against bacteria, fungi and viruses at mucosal surfaces (Naser *et al.*, 2012). Moreover, $CD4^+$ T cells secreting interleukin-17 (Th-17 cells) have been previously implicated in IBD as well as colitis in animal models associated with an up-regulated T helper type 1 response (Duerr *et al.*, 2006; Kaser & Blumberg, 2008). Several genes in the Th-17 pathway have been attributed to IBD susceptibility, including *IL23R, TNFSF15, STAT3, IL12B, CCR6* and *JAK2*; however *IL23R* confers the highest risk for CD development (Duerr *et al.*, 2006; Frank, 2007; Hampe *et al.*, 2007; Libioulle *et al.*, 2007; Pidasheva *et al.*, 2011; Rioux *et al.*, 2007; Yamazaki *et al.*, 2005). IL23-R, located on chromosome 1p31, is a protein consisting of IL-12β1 and an IL-23R chain and is highly expressed on activated memory T-cells as well as natural killer cells, monocytes, and dendritic cells (Tsianos *et al.*, 2012). IL-23 is a key pro-inflammatory cytokine and signals through the heterodimeric receptor complex IL-12β1/IL-23R (Gazouli *et al.*, 2010). Upon binding of IL-23 to IL-23R, STAT3 is phosphorylated leading to IL-23 gene expression. Interestingly, in humans it has been shown that IL-23 is over expressed in clinical samples of CD (Di Meglio *et al.*, 2011; Schmidt *et al.*, 2005).

A variant of IL23-R, rs11209026 (R381Q) was identified for conferring strong protection against the development of CD (Libioulle *et al.*, 2007). Moreover, this polymorphism is significantly higher among healthy controls than in patients (Di Meglio *et al.*, 2011). The R381Q variant is characterised by an arginine (R) to glutamine (Q) substitution at position 381. The R381Q polymorphism is located between the trans-membrane domain and the putative JAK2 binding site in the cytoplasmic portion of IL23R protein, and is highly conserved across different species (see fig. 1.5) (Pidasheva *et al.*, 2011). This polymorphism leads to a decreased number of IL-23 responsive cells and as a consequence diminished IL-23 and IL-17A activity (Pidasheva *et al.*, 2011).

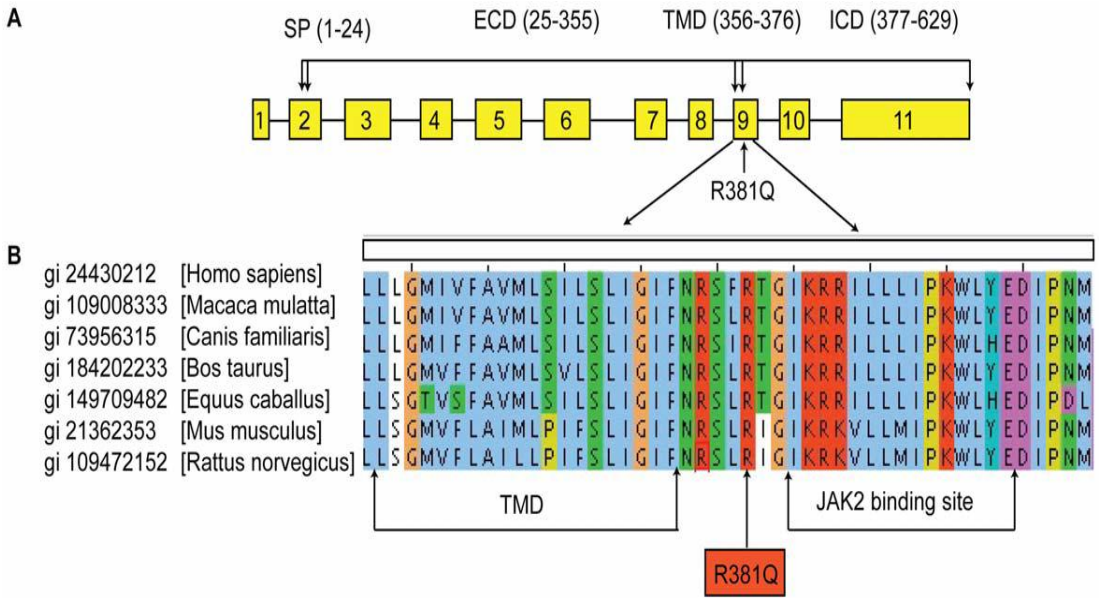


Figure 1.5: The R381Q polymorphism (arginine at 381) of the IL23R is highly conserved across different species.

(A) Map of the IL23R gene. (B) Sequence alignment of IL23R protein sequences from different species (from Gazouli *et al* 2011). The rs11209026 (R381Q) variant in the *IL23* gene on chromosome 1p31 may act as possible protective variant against Crohn's disease.

The rare R381Q polymorphism was significantly underrepresented in both childhood-onset and adult-onset compared to control patients (Amre *et al.*, 2008; Dubinsky *et al.*, 2007; Gazouli *et al.*, 2010). Due to the role of IL-23 in activation of inflammatory responses, blocking this pathway may lead to a good therapeutic approach for autoimmune disorders such as CD (Libioulle *et al.*, 2007). Additional studies have also reported the association of the *IL23R* gene to CD in North American and European cohorts (Fujita *et al.*, 2008).

1.1.5 Environmental risk factors for Crohn's disease

Many environmental and lifestyle factors have been implicated in CD such as smoking, oral contraceptives (Corrao *et al.*, 1998) and it has even been suggested that lack of breastfeeding during infancy might influence the onset of IBD later in life (Bergstrand & Hellers, 1983).

Varying patterns of cigarette smoking in patients with IBD have been observed. For example, smoking is strongly associated with CD (Calkins, 1989); however, in contrast epidemiological studies have shown an inverse correlation between the prevalence of smoking and the incidence of UC, which is said to be largely a disease of non-smokers and former smokers (Birrenbach & Bocker, 2004; Corrao *et al.*, 1998; Tobin *et al.*, 1987). Moreover, smoking may even result in a beneficial influence on the course of ulcerative colitis (Birrenbach & Bocker, 2004). In a previous study examining siblings with similar genetic susceptibility for IBD, smokers tended to develop CD whereas non-smokers were more prone to developing UC. It is important to note that this study did not examine genetically identical sibling pairs and therefore gene differences between them could still influence the disease that may develop (Bridger *et al.*, 2002). Among the elements contained in tobacco, it is clear that nicotine has the greatest impact upon the clinical course of CD (Galeazzi *et al.*, 1999; Nos & Domenech, 2011). The potential mechanisms hypothesised to be influenced by nicotine include changes in humoral and cellular immunity, cytokine and eicosanoid levels, gut motility, permeability, and blood flow (Birrenbach & Bocker, 2004). Smokers show high levels of reactive oxygen species and a lessened antioxidant capacity which could act synergically with oxidative

stress in CD (Beltran *et al.*, 2009; Nos & Domenech, 2011). The relationship between CD and smoking was first observed in 1984 in a case-control study of 82 CD patients and matched controls drawn from general practice lists. They observed that patients with CD were significantly more likely to be smokers than control patients and the association was stronger for smoking habit before the onset of the disease than for current smoking habit, the relative risks for smokers compared with non-smokers being 4.8 and 3.5 respectively (Somerville *et al.*, 1984). Further studies have also confirmed the initial findings (Bridger *et al.*, 2002; Calkins, 1989; Corrao *et al.*, 1998). Smoking has also been associated with a worse prognosis of CD and CD patients who continue to smoke after diagnosis have an increased risk of relapse as well as increased pain and an increased probability of hospital emissions (Holdstock *et al.*, 1984). Moreover, from a study on a Canadian cohort of 152 CD patients, the relapse rate was higher among smokers (Timmer *et al.*, 1998). Smoking has been shown to have an effect on macrophages by altering alveolar macrophage recognition and phagocytic ability (Hodge *et al.*, 2007). However, the harmful effect of active smoking is not uniform in all patients or in all clinical scenarios and further study needs to be carried out. It is important to note that smoking may act on a specific genetic background, where the risks between CD and UC are evenly balanced, to influence whether the outcome is CD in smokers or UC in non-smokers (Bridger *et al.*, 2002).

Studies have also reported that diet can also contribute to CD. An imbalance in the consumption of high in saturated fats, processed foods and high sugar foods may be linked to the occurrence of CD, since CD is more common in the developed world (Asakura *et al.*, 2008). It was originally believed that the composition of the intestinal microbiota was relatively stable from early childhood; however, recent evidence suggests that diet can cause dysbiosis, an alteration in the composition of the microbiota, which could lead to aberrant immune responses (Brown *et al.*).

1.1.6 Bacterial involvement in Crohn's disease

As previously mentioned, the most widely held hypothesis on the pathogenesis of CD is an aggressive hyper-active acquired Th-1 and Th-17 immune responses to a subset of commensal enteric bacteria in a genetically susceptible host with

environmental factors contributing to the onset or reactivation of CD. In order for disease to become clinically apparent it was thought that some of these factors must interact (Sartor, 2006). CD is characterized by increased production of IL-12, IL-23, IL-27, interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α (Sartor, 2006). Over the past decade there has been growing evidence to support the notion that indigenous intestinal flora is an important target of the immune response underlying IBD (Barnich & Darfeuille-Michaud, 2007b; Eckburg & Relman, 2007; Fiocchi, 2005; Lodes, 2004).

The complex, predominantly anaerobic microbiota in the distal ileum and colon can act as a constant source of antigens and adjuvants that can stimulate chronic immune-mediated inflammation in genetically susceptible hosts (Balfour Sartor, 2007). Bacterial adjuvants such as lipopolysaccharide, peptidoglycan, flagellin and non-methylated DNA (CpG motif), can bind to various Toll-like receptors (TLRs) on innate immune cells and epithelial cells resulting in the activation of innate immune responses and consequently, the up-regulation of IL-1β, TNF-α, IL-6, IL-8 and other chemokines such as IL-12 p40 which are all involved in the inflammatory response (Sartor, 2006). Bacterial antigens can also activate the adaptive immune response and stimulate the clonal expansion of T cells that selectively recognize the antigen through their T-cell receptor (Sartor, 2006). Either process can lead to overly aggressive T-cell responses to normal bacteria resulting in tissue damage (see fig 1.6)**.**

Experimental data from animal models of IBD show that components of the microbial flora are necessary to develop colitis. Symptoms of IBD fail to develop if animals are kept under germ-free (gnotobiotic) conditions (Fiocchi, 2005; Frank, 2007; Strober *et al.,* 2002). Moreover, in some cases patients with CD experience improvement in clinical disease when administered prolonged courses of antibiotics such as oral ciprofloxacin and rifaximin (Arnold *et al.,* 2002; Eckburg & Relman, 2007; Gionchetti *et al.,* 2005). Additionally, clusters of cases of CD over time suggest an infectious etiology (Van Kruiningen *et al.,* 1993) and some of the pathological features of CD, such as aphthous ulcers of the mucosa, mural abscesses,

macrophage and epithelioid cell granulomas and macrophage recruitment are also associated with other intestinal diseases involving *Shigella* spp., *Salmonella* spp., *Yesinia enterocolitica* and *Mycobacterium tuberculosis,* all of which may indicate an immune reaction to microbes or microbial antigens/adjuvants (Meconi, 2007).

1.1.6.1 Defective mucosal barrier function and microbial clearance

The intestinal epithelium represents a highly selective barrier between the gut lumen and underlying cells of the immune system. Intestinal epithelial cells must adapt to constant changes in their environment by processing both bacterial and host-derived immune signals (Werner *et al.*, 2010). A defective mucosal barrier could lead to increased uptake of luminal antigens/adjuvants overwhelming the mucosal immune response leading to constant stimulation (Sartor, 2006). As previously mentioned, each of the polymorphisms associated with CD, including *NOD2/CARD15* and *IBD5* are involved in epithelial function. Immunohistochemical studies have shown the presence of *Listeria*, *Streptococcus* species and *E. coli,* in CD mucosal macrophages (Liu *et al.*, 1995). Furthermore, high concentrations of immunoglobulin G (IgG) directed against commensals have been shown in CD patients indicating a defective mucosal barrier function or a breakdown in tolerance to normal gut commensals (Eckburg & Relman, 2007; Macpherson *et al.*, 1996). Given the intracellular location of NOD2/CARD15 and its role in stimulating defensins and mediating intracellular bacterial killing, the presence of invasive bacteria may be an important link between the innate immune response and the development of inflammation associated with CD (Rolhion, 2007).

In mouse models, when the intestinal barrier is defective due either to altered tight junctions or removal of enteric glial cells, severe gut inflammation is observed (Bush *et al.*, 1998; Hermiston & Gordon, 1995). Additionally, certain bacteria such as *E. coli* have been shown to increase bowel permeability in animal models (Garcia-Lafuente *et al.*, 2001). Altered intestinal permeability has been observed in CD patients and interestingly, polymorphisms in the *IBD5* gene, whose products play a role in epithelial cell polarity may potentially impact on intestinal permeability (Eckburg & Relman, 2007; Peltekova *et al.*, 2004). It has also been demonstrated that mucus which is an integral part of the mucosal barrier may play an important

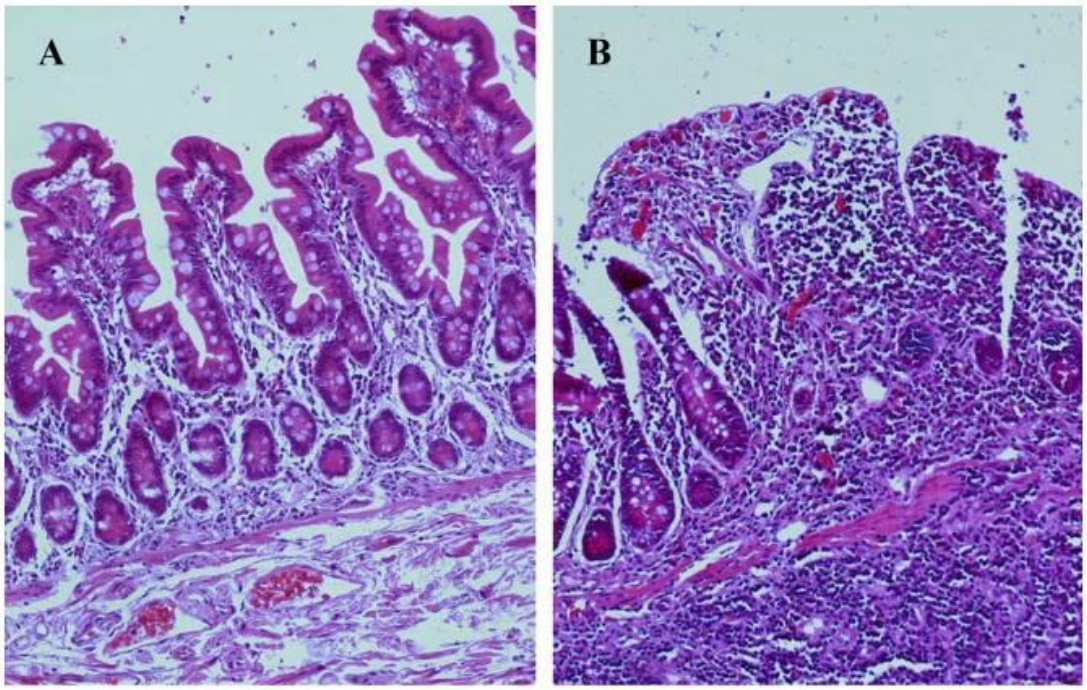


Figure 1.6 Histological features of the ileal mucosa from a CD patient.

A) H&E staining from a healthy patient showing normal epithelial phenotype with few infiltrated inflammatory cells. B) Mucosal specimen from CD patient showing disrupted mucosal architecture and the infiltration of inflammatory cells. Magnifications: x100 (Qiurong L *et al* 2012)

role in by stopping penetration of the mucosa by luminal bacteria and their products thus preventing the inflammatory process (Fyderek *et al.*, 2009). A previous study showed that a reduction of mucus layer thickness in adult IBD patients caused increased exposure of the microbes to the gut immune system, which in turn increased inflammation (Pullan *et al.*, 1994). However, this was primarily associated with UC rather than CD. In this study many CD patients showed an increased mucus thickening.

1.1.6.2 Microbial diversity

The colonic microbiota is composed of hundreds of bacterial species and sub-species and a close metabolic relationship exits exists between these microbes and their host. The gut microbial content varies dramatically between individuals, with each individual harbouring a stable and unique bacterial population over time (Fava & Danese, 2011). Modern molecular techniques have shown that CD faecal samples have a marked reduction in diversity (Friswell *et al.*, 2010; Manichanh *et al.*, 2006). Such studies have shown that in healthy individuals, approximately 95% of the bacteria in stool samples belong to the bacteroides and the Firmicutes (Friswell *et al.*, 2010; Suau *et al.*, 1999). However, a marked reduction in the numbers of these organisms was observed in IBD patients (Suau *et al.*, 1999). Furthermore, studies of the bacterial composition in the lumen of CD patients showed a decrease in the amount of beneficial bacteria, such as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* (Barnich & Darfeuille-Michaud, 2007a; Mondot *et al.*, 2011). A strong association has been shown between the latter firmicute and ileal CD. One hypothesis is that butyrate producers such as low mol% G+C content gram-positive colonic bacteria like *F. prausnitzii*, promote gastrointestinal tract health, as butyrate is an important source of energy for colonic epithelial cells and also has anti-inflammatory effects (Louis, 2007). Moreover, butyrate reinforces the mucosal barrier by inducing mucin production and antimicrobial peptides. Therefore decreased levels of butyrate could contribute to the inflammation of IBD (Fava & Danese, 2011). *F. prausnitzii*, despite being extremely oxygen sensitive can survive in the gut mucosa where oxygen readily diffuses from epithelial cells. This paradox was recently explained on the basis that *F. prausnitzii* employs an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen, a novel host

microbe interaction at the gut level (Khan *et al.*, 2012). Interestingly, patients with CD have been reported to show higher numbers of mucosa-associated bacteria and a thicker mucosal layer of bacteria then control patients (Seksik P, 2006; Swidsinski *et al.*, 2002). In a previous study, when the overlying mucus layer was removed from a healthy patient, the underlying normal colonic mucosa was relatively free from aerobic bacteria, whereas the CD mucosae contained a significant amount of aerobic flora (Martin *et al.*, 2004). Additionally, a number of studies have reported a less diverse ecosystem and an increase in the abundance of *Enterobacteriaceae*, particularly *E. coli* as well as other *Bacteroides* and *Enterococcus* species in CD patients compared to the diverse collection of *Firmicutes* (low %GC gram-positive bacteria) observed in healthy controls (Friswell *et al.*, 2010; Sartor, 2009; Seksik P, 2006). Similar results were observed in a recent study on a Chinese cohort, which showed for the first time the faecal bacterial dysbiosis in Chinese CD patients was characterized by an increase of the richness γ-Proteobacteria (especially *E. coli* and *Shigella flexneri*) and a reduced proportion of *Firmicutes* (Li *et al.*, 2012). An altered balance of beneficial versus aggressive microbial species could lead to chronic inflammatory response in a susceptible host and such studies indicate a possible relationship between some bacteria and the pathology of CD.

1.1.6.3 Association of specific microbes with Crohn's disease

Over the years several studies have implicated different microorganisms in the pathogenesis of CD (see table 1.1) although it is important to note no consensus opinion exists for any one microorganism as the cause of CD.

Table 1.1 Key microorganisms associated with the pathogenesis of Crohn's disease (modified from Eckburg, P.B and Relman, D.A 2007)

Organisms	References
Adherent Invasive *Escherichia coli* (AIEC)	(Claret, 2007; Darfeuille-Michaud, 2002; Darfeuille-Michaud, 2004; Darfeuille-Michaud, 1998; Lapaquette *et al.*, 2012a; Martin *et al.*, 2004; Martinez-Medina *et al.*, 2009a)
Mycobacterium avium subspecies *paratuberculosis* (MAP)	(Autschbach *et al.*, 2005; Chiodini *et al.*, 1984; Naser *et al.*, 2004; Sartor, 2005)
Measles virus	(Ekbom *et al.*, 1994; Smith & Wakefield, 1993)
Saccharomyces cerevisiae	(Mow *et al.*, 2004)
Enterococcus species	(Sartor, 2006)
Helicobacter pylori	(D'Inca *et al.*, 1998; Puspok *et al.*, 1999)

Numerous studies have implicated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in the etiology of CD (Autschbach *et al.*, 2005; Chiodini *et al.*, 1984; Naser *et al.*, 2004; Sartor, 2005). Granulomatous inflammation associated with CD closely resembles that of an enteritis in ruminants, known as Johne's disease, the causative agent of which is recognised to be MAP (Harris & Barletta, 2001). The notion that MAP is linked to CD was first proposed by Dalziel almost 100 years ago (Dalziel, 1913). In the mid-1980s strains of atypical mycobacteria cultured from three CD-affected intestinal specimens were identified as MAP (Chiodini *et al.*, 1984). Non-controlled studies suggested that up to 85% of CD patients responded to antibiotics effective against MAP (Sartor, 2006). However, numerous groups have attempted to culture MAP from CD affected tissue with inconsistent and conflicting results, MAP detection rates have ranged from 0–100% (Sartor, 2006). Moreover, MAP and its DNA have been repeatedly detected in healthy controlled tissue casting doubt on its role in CD (Eckburg & Relman, 2007).

Atypical mycobacteria are ubiquitous in the environment and MAP was detected using IS900 (the insertion sequence of MAP) in commercial pasteurized milk and in human breast milk, providing a mode of transmission (Millar *et al.*, 1996). As findings are inconsistent among research groups, the role of these pathogens in CD is inconclusive. It has been suggested that the common environmental agent MAP selectively colonizes or lodges in the ulcerated mucosa of CD patients but is not the causative agent of CD (Sartor, 2006). MAP could however, act as an environmental factor by increasing AIEC intramacrophage replication via decreasing macrophage function (Mpofu *et al.*, 2007).

1.1.6.4 Crohn's disease-associated *E. coli*

Recently, compelling and reproducible evidence supporting a role for *E. coli* in ileal CD has been presented. *E. coli* is the predominant facultative anaerobic gram negative species of the intestinal flora and plays an important role in maintaining normal intestinal conditions. A previous immunohistological study observed that approximately 69% of CD mucosal samples were positively labelled with polyclonal *E. coli* antibodies (Cartun *et al.,* 1993). Furthermore, CD patients have been shown to have higher *E. coli* antibody titers than healthy individuals (Bringer, 2006).

Increased numbers of mucosa-associated *E. coli* are observed in CD patients (Darfeuille-Michaud, 1998; Neut *et al.*, 2002). It has been reported that adhesive *E. coli* were isolated from 62% of CD patients compared to only 6% of healthy controls (Giaffer *et al.*, 1992) and interestingly, *E. coli* expressing adherence factors were more readily isolated from CD and UC patients compared to normal controls (Kotlowski *et al.*, 2007). This suggests that adherence factors of *E. coli* may have a significant role in disease aetiology. Higher concentrations of bacteria allow for biofilm development on the surface of the gut mucosa of patients with CD (Rolhion, 2007). This property could enable the bacteria to colonize the intestinal mucosa. Additionally, clusters of *E. coli* were observed in the lamina propria in CD samples but not in healthy controls and *E. coli* were isolated more frequently from the intestinal serosa of CD patients compared to controls (Ambrose *et al.*, 1984; Laffineur *et al.*, 1992). Moreover, a study using laser capture microdissection (LCM) and PCR detected the presence of *E. coli* in granulomas from 12 (out of 15) CD

patients, in comparison with only 1 from, 10 non-CD granuloma control patients (Claret, 2007). Interestingly, unusually high amounts of anti- *E. coli* outer membrane porin C (anti-OmpC) antibodies have been detected in CD patients compared to healthy controls. Anti-OmpC antibiodies have been identified in 37%-55% of CD patients in comparison to less than 5% of healthy controls (Landers *et al.*, 2002; Mei *et al.*, 2006). Significantly, reactivity to *E. coli* OmpC is associated with more severe forms of CD resulting in frequent disease progression and longer disease duration (Landers *et al.*, 2002). Such studies have led to the description of a new pathotype of *E. coli*; adherent-invasive *Escherichia coli* (AIEC) that is associated with CD and other inflammatory conditions such as granulomatous colitis in boxer dogs (Simpson *et al.*, 2006).

1.2 *Escherichia coli*: commensals and pathogens

As previously mentioned *E. coli* are Gram-negative bacilli from the family *Enterobacteriaceae* and is the dominant facultative anaerobe present in the human intestinal flora. Therefore *E. coli* is commonly found as a commensal organism colonizing the mucous layer of the mammalian colon and small intestine (Boudeau *et al.*, 1999). The mechanisms whereby *E. coli* assures its favourable niche in the colon and small intestine are poorly understood, however one hypothesis suggests *E. coli* exploits its ability to utilise gluconate more efficiently than other microbes present in the colon and this allows it to metabolise and dominate effectively in this niche (Kaper, 2004).

E. coli colonizes the gastrointestinal tract of human infants shortly after birth. Normally, as a commensal *E. coli* co-exists with its human host in good harmony and only causes disease in immunocompromised hosts or when the intestinal barrier is breached (Rolhion, 2007). However, some *E. coli* have acquired different sets of virulence genes via horizontal transfer of DNA on plasmids, transposons, bacteriophages and pathogenicity islands (PAI) allowing them to adapt to new niches and cause a broad spectrum of diseases (see fig 1.7) (Kaper, 2004; Nataro & Kaper, 1998). Additionally, commensal *E. coli* can also undergo point mutations or deletions that contribute to virulence in these strains (Kaper, 2004). Pathogenic *E.*

coli can be therefore defined on the basis of their pathogenic mechanisms (known genes contributing to phenotypic characteristics and pathogenesis).

1.2.1 Pathotypes of *Escherichia coli*

Pathogenic *E. coli* can be classified as either extraintestinal *E. coli* (ExPEC) or diarrhoeagenic *E. coli*. ExPEC can be subdivided into two pathotypes – uropathogenic *E. coli* (UPEC) and neonatal meningitis *E.coli* (NMEC). Within the diarrhoeagenic group, six pathotypes exist: enterohaemorrhagic *E.coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper, 2004; Nataro & Kaper, 1998; Rolhion, 2007). An additional animal pathotype, known as avian pathogenic *E. coli* (APEC), causes extraintestinal infections; primarily respiratory infections and septicaemia of poultry (Kaper, 2004). The various pathotypes of *E. coli* tend to be clonal groups characterised by shared O- (lipopolysaccharide) and H- (flagellar) antigens (Kaper, 2004).

Colonisation is mediated by adhesins on the surface of bacteria, which allow the bacteria to bind to specific receptors on the host cell. For example, *Salmonella* spp. can adhere to follicle-associated epithelial cells with the help of multiple fimbriae or pili (rod-like structures of 5-10 nm diameter that are distinct from flagella), some of which are encoded by the *lpf*, *pef* and *agf* operons (Detweiler, 2003). *E. coli* typically express filamentous surface adhesive organelles known as Type 1 fimbriae (Type IV pili or Pap pili may also be present in UPEC). It has been shown, that the distal tip of Type 1 fimbriae contains an adhesin protein known as FimH. FimH recognises mannose and mannose-containing glycoproteins and has been shown to mediate UPEC attachment to host bladder cells (Eto, 2008). Adhesins of some pathogenic *E. coli* include non-fimbrial proteins e.g. afa and outer membrane proteins such as intimin of EPEC and EHEC. Ultimately, adhesion enables the bacteria to effectively colonise the intestinal mucosa and to resist mechanical removal (Rolhion, 2007).

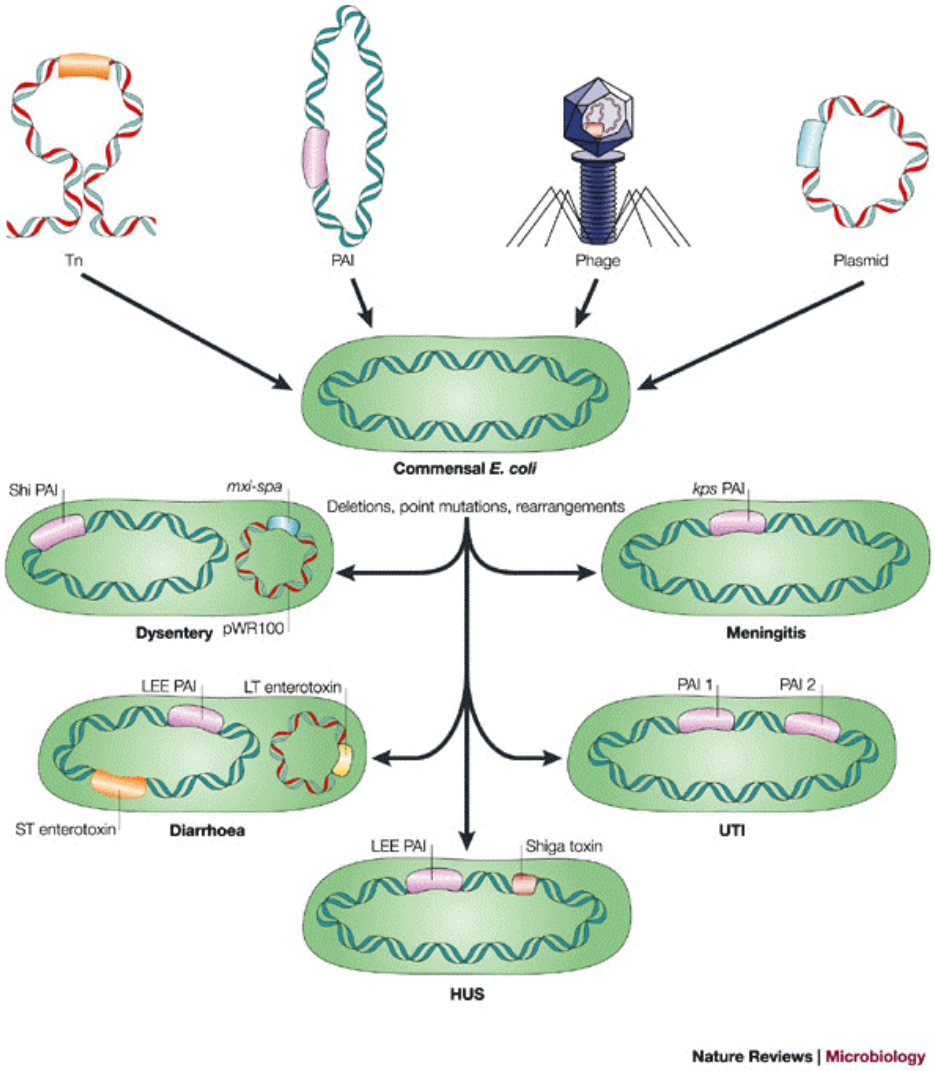


Figure 1.7 Mobile genetic elements contributing to the evolution of pathogenic *E. coli*.

E. coli virulence factors are encoded by a number of mobile genetic elements, including transposons (Tn), bacteriophage, plasmids and pathogenicity islands (PAI). Commensal *E. coli* can also undergo deletions resulting in point mutations or other DNA rearrangements that can contribute to virulence. These additions, deletions and other genetic changes can give rise to *E. coli* pathotypes capable of causing diarrhoea (EPEC, EHEC, EAEC, DAEC), dysentery (EIEC), haemolytic uremic syndrome (EHEC), urinary tract infections (UPEC) and neonatal meningitis *E.coli* (NMEC) (Kaper *et al* 2004).

1.2.1.1 Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of *E. coli* to be described and it is linked to infant diarrhoea in the developing world. Moreover, numerous case-control studies on six continents have found EPEC to be more frequently isolated from infants with diarrhoea than adults. EPEC infection is characterised by its hallmark intestinal histopathology; known as 'attaching and effacing (A/E)' which can be observed in both intestinal biopsy samples from patients and in cell culture (Andrade *et al.*, 1989; Donnenberg *et al.*, 1989; Donnenberg *et al.*, 1990; Jerse *et al.*, 1990). This phenotype involves the bacteria intimately attaching to intestinal epithelial cells and inducing marked cytoskeletal changes such as accumulation of polymerized actin under the adherent bacteria. Consequently, the microvilli of the intestinal epithelial cells are effaced and the bacteria usually sit upon a raised pedestal-like structure (Moon *et al.*, 1983). Pedestal formation is a dynamic process as video microscopy studies have shown that these EPEC pedestals can bend and stretch while remaining in place on the epithelial cell surface (Sanger *et al.*, 1996). Localized adherence of EPEC to epithelial cells is mediated by 7-nm diameter type IV fimbriae which aggregate and form bundles thereby acquiring the name "bundle-forming pilus" (BFP) (Giron *et al.*, 1991). The genes for BFP are located on a 70-100kb plasmid called the EAF (EPEC adherence factor) plasmid (Nataro & Kaper, 1998). Subsequent genetic studies have revealed that 13 genes on the EAF plasmid are required for the expression and assembly of BFP, many of which encode proteins showing homology to proteins required for type IV pilus assembly in *Pseudomonas aeruginosa* (Sohel *et al.*, 1996).

The ability to induce the characteristic A/E phenotype is due to a collection of genes located on 35-kb pathogenicity island (PAI) known as the locus of enterocyte effacement (LEE) which encodes a type III secretion system, multiple secreted proteins, and the bacterial adhesin intimin (McDaniel *et al.*, 1995; Nataro & Kaper, 1998). Following adhesion, the type III secretion system is activated and various effectors such as Tir, EspF, EspG EspH and MAP are translocated into the host cell. Furthermore, Tir (translocated intimin receptor) is shown to be inserted into the host-cell membrane, where it functions as a receptor for the intimin outer-membrane protein (Kenny *et al.*, 1997). EPEC can now bind intimately with the epithelial cell

due to the interaction of intimin with Tir. Secretion of effectors resulting in numerous cytoskeletal changes and tight junctions loosen leading to increase permeability. Diarrhoea occurs due to microvillus effacement resulting in active ion secretion, increased intestinal permeability and intestinal inflammation (Kaper, 2004).

1.2.1.2 Enterohaemorrhagic *E. coli* (EHEC)

EHEC causes bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhoea and haemolytic uremic syndrome (HUS). EHEC can be found in the fecal flora of a wide variety of animals including sheep, goats, pigs, cats and dogs however, the most important reservoir regarding human infection is cattle (Burnens *et al.*, 1995). A wide variety of food items are associated with EHEC infection including hamburgers, sausages, unpasteurised milk, lettuce, apple juice and radish sprouts (Beutin & Martin, 2012; Gaulin *et al.*, 2012; Xiong *et al.*, 2012). The most important EHEC pathogens are associated with the O157:H7 serotype and this serotype is most commonly associated with outbreaks in North America, the United Kingdom and Japan (Kaper, 2004). EHEC contains the LEE pathogenicity island but the key virulence factor and defining characteristic for EHEC is the production Stx (Shiga toxin) also known as verocytotoxin (VT). This potent cytotoxin is the main factor that leads to death and many other symptoms associated with EHEC infection. Stx is encoded on a bacteriophage inserted into the chromosome of *E. coli* (O'Brien *et al.*, 1984). The Stx family contains two subgroups Stx-1 and Stx-2 and a single EHEC strain can possess, Stx1, Stx2 (Nataro & Kaper, 1998). Stx-1 differs from Stx by only 1 amino acid. Stx-2 shares 56% sequence homology with Stx-1 (Nataro & Kaper, 1998). Stx is a complex A-B toxin consisting of a single A subunit that cleaves ribosomal RNA disrupting protein synthesis and 5 identical B subunits that allow the toxin to bind to glycolipid globotriaosylceramide (Gb3) on the target host cell surface (Khan *et al.*, 2003). The resulting disruption of protein synthesis leads to critical damage of renal endothelial cells, apoptosis of intestinal epithelial cells, HeLa cells, or any cells which possess the Gb3 receptor (Nataro & Kaper, 1998).

Following infection, Stx is produced in the colon and migrates, via the bloodstream, to the kidney. Here, it can damage renal endothelial cells and induce production of

local chemokines and cytokines resulting in inflammation. If severe, this damage can lead to HUS characterized by haemolytic anaemia and in some cases fatal acute renal failure (Andreoli *et al.*, 2002). EHEC strains also contain the LEE pathogenicity similar to EPEC and the classic intestinal histopathology characteristic of *E. coli* O157:H7 infection includes haemorrhage and swelling in the lamina propria (Griffin *et al.*, 1990). The genome sequence of O157:H7, which was the first reported genome sequence of a pathogenic *E. coli*, revealed numerous chromosomal islands that encode additional potential virulence factors such as novel fimbriae and iron uptake systems (Perna *et al.*, 2001).

1.2.1.3 Enterotoxigenic *E. coli* (ETEC)

ETEC strains are associated with both childhood diarrhoea in the developing world and diarrhoea in travellers to developing countries (Nataro & Kaper, 1998). The diarrhoea is usually watery without blood. ETEC colonises the surface of the small bowel mucosa and colonisation is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CFs), which are designated by CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) (Kaper, 2004). Over 70% of human ETEC express either CFA/I, CFA/II or CFA/IV although more than 20 different CFs have been identified (Wolf, 1997). ETEC produces enterotoxins that belong to one of two groups: (i) the heat-labile enterotoxin (LT) or (ii) the heat-stable enterotoxin (ST). CFA genes are usually encoded on plasmids, which typically also encode the enterotoxins ST and/or LT (Nada *et al.*, 2011) and ETEC strains cause diarrhoea through the action of the enterotoxins. LT enterotoxins, predominantly found in human isolates are closely related to the cholera enterotoxin (CT) in *Vibrio cholerae* (Hicks *et al.*, 1996). LT is a complex A-B toxin consisting of a single A subunit and five identical B subunits. The A subunit is responsible for the enzyme activity of the toxin whereas the mediate binding of the toxin to the host cell surface gangliosides GM1 and GD1b (Kaper, 2004).. The A subunit has ADP-ribosyltransferase activity and acts by transferring an ADP-ribosyl moiety from NAD+ to the alpha subunit of the GTP-binding protein, G_S resulting in stimulation of adenylate cyclase activity. This leads to increased levels of intracellular cyclic AMP (cAMP) and the activation of the main chloride channel of epithelial cells, the cystic fibrosis transmembrane

conductance regulator (CTFR). The net result is inhibition of NaCl absorption by villus tip cells and increased water content ultimately resulting in osmotic diarrhoea (Sears & Kaper, 1996). In contrast to LT, ST is a small, single peptide toxin that contains multiple cysteine residues. The resulting disulfide bonds account for the heat stability of these toxins (Nataro & Kaper, 1998). There are two unrelated classes of STs that differ in both structure and function: (i) STa and (ii) STb and genes for both classes are predominantly found on plasmids and on transposons (Nataro & Kaper, 1998). Only the STa class has been associated with human infection and the main receptor for STa is a membrane-spanning guanylate cyclase. Binding of STa leads to increased guanylate cyclase activity and increased cGMP which, in turn, activates cGMP-dependent and/or cAMP-dependent kinases. All of which ultimately leads to increased secretion and watery diarrhoea (Kaper, 2004).

1.2.1.4 Enteroaggregative *E. coli* (EAEC)

EAEC are the cause of persistent diarrhoea of children and adults in both the developing and developed countries. EAEC strains characteristically enhance mucus secretion from the mucosa which traps the bacteria in a thick bacterium-mucus biofilm as a 'stacked brick' configuration adhering loosely to the mucosal surface (Nataro & Kaper, 1998). The pathogenesis of EAEC is not well understood although the basic strategy appears to be colonisation of the mucosa of the colon followed by secretion of enterotoxins and cytotoxins (Nataro *et al.*, 1998). EAEC adhesion is mediated by fimbrial structures known as aggregative adherence fimbriae (AAFs) although it should be noted that not all EAEC strains adhere via AAFs (Czeczulin *et al.*, 1997; Nataro *et al.*, 1992). Many EAEC strains also secrete an autotransporter toxin called Pet encoded on the same virulence plasmid as AAF. This autotransporter could potentially be involved in cytoskeletal changes in epithelial cells (Navarro-Garcia *et al.*, 1998). Following adhesion, enhanced mucus production may occur, leading to a deposition of a thick mucus containing biofilm encrusted with EAEC (Nataro & Kaper, 1998). A number of toxins have been described for EAEC such as the oligomeric enterotoxin *Shigella* enterotoxin 1 (ShET1). Although the mode of action of ShET1 is poorly understood it may contribute to damage to intestinal cells and watery diarrhoea exhibit by EAEC and *Shigella* infections (Noriega *et al.*, 1995). Interestingly, a recent outbreak that comprised 3,801 cases of human infections with

an emerging *E. coli* pathotype enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) O104:H4 occurred in Germany in May 2011 with a further 100 cases reported in 12 other countries (Mellmann *et al.*, 2011). EAHEC strains have evolved from EAEC by acquisition of a Shiga toxin 2a (Stx2a)-encoding bacteriophage. This is a clear example of how the acquisition of mobile genetic elements can lead to the evolution of pathogenic traits in *E coli*.

1.2.1.5 Enteroinvasive *E. coli* (EIEC)

EIEC are biochemically, genetically and pathogenically related to *Shigella* spp. Both organisms invade the colonic epithelium mediated by both plasmid and chromosomal loci. Moreover, both organisms produce one or more secretory enterotoxins that may play roles in diarrheal pathogenesis (Nataro & Kaper, 1998). However, due to the clinical significance of *Shigella* a nomenclature distinction has been maintained (Kaper, 2004). EIEC elicits watery diarrhoea that is indistinguishable from infections by other pathogenic *E coli* (Nataro & Kaper, 1998). The initial phase of EIEC/*Shigella* infection involves epithelial cell penetration, followed by lysis of the endocytic vacuole. Following this, intracellular multiplication occurs and the bacterium undergoes directional movement through the cytoplasm and extends into neighbouring adjacent epithelial cells. This movement with the cytoplasm is mediated by nucleation of cellular actin which extends from one pole of the bacterium as a "tail" and allows for bacterial movement through the cell (Sansonetti, 2002). VirG (IcsA) is a surface protein found to be essential for nucleation of actin filaments allowing movement (Goldberg *et al.*, 1993).. Genes necessary for invasiveness are carried on a 140-MDa plasmid, known as pInv, in EIEC and other *Shigella* serotypes. This plasmid encodes a type III secretion system which secretes multiple proteins, such as IpaA, IpaB, IpaC and IpgD, which are effectors of the invasive phenotype and mediate epithelial signalling events, cytoskeletal rearrangements, lysis of the endocytic vacuole and other actions (Sansonetti *et al.*, 2000). Prominent genes also located on this plasmid are the *mxi* and *spa* loci, which encode the type III secretion apparatus. This enables the insertion of a pore containing IpaB and IpaC proteins into host cell membrane. IpaC has been shown to promote the uptake of *Shigella* spp. into the eukaryotic cell (Marquart *et al.*, 1996) and IpaB is thought to function in the lysis of the phagocytic

vacuole (Nataro & Kaper, 1998) and in the induction of apoptosis in macrophages (Zychlinsky *et al.*, 1994).

1.1.4.1.5 Diffusely adherent *E. coli* (DAEC)

The term "diffusely-adherent *E coli*" refers to the characteristic diffuse pattern of adherence to HEp-2 cell monolayers (Nataro *et al.*, 1987). A number of studies have shown that DAEC induce finger-like projections extending from the surface of infected Caco-2 or HEp-2 cells (Cookson & Nataro, 1996; Yamamoto *et al.*, 1994). DAEC have been implicated as the cause of diarrhoea in a number of studies primarily affecting children under the age of 12 (Scaletsky *et al.*, 2002). Some DAEC express a surface fimbrial adhesin which mediates the diffusely-adherent (DA) phenotype. This fimbrial adhesin, known as F1845 is found in approximately 75% of DAEC and these fimbrial genes show homology to members of the Afa/Dr group of bacterial adhesins (Servin, 2005). Other DEAC express afimbrial adhesin (Afa). Afa/Dr adhesins utilise decay-accelerating factor (DAF), a cell-surface glycosylphosphatidylinositol- anchored protein, which normally protects cells from damage by the complement system, as the receptor (Kaper, 2004). Dr fimbriae cluster at the DAF receptor and lead to the activation of signal transduction cascades (Peiffer *et al.*, 1998). Afa/Dr adhesins also recognise the membrane bound receptors type IV collagen, CEACAM1, CEA, and CEACAM6 which lead to proinflammatory responses in polarized intestinal cells (Servin, 2005). Moreover, DAEC expressing the fimbrial adhesion F1845 induce dramatic changes in the architecture of the microvilli (limited to the point of bacterial contact with the microvilli, showing disruption and then nucleation) and induce apical F-actin disorganization without bacterial entry (Peiffer *et al.*, 1998).

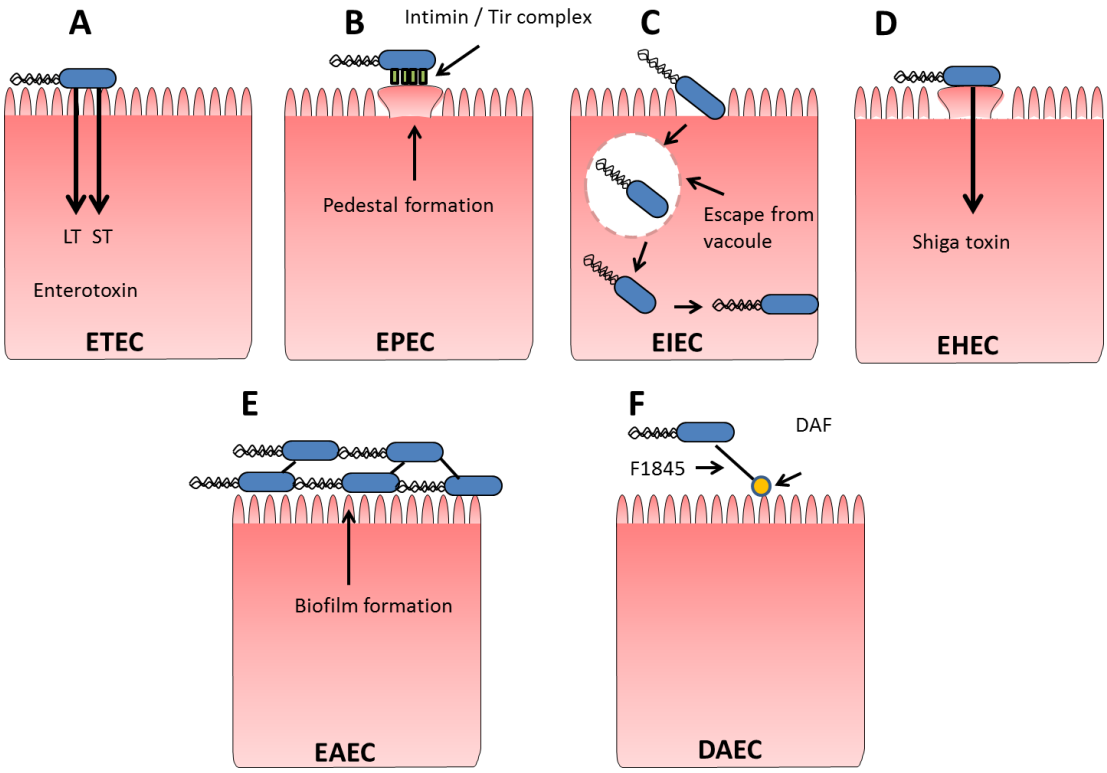


Figure 1.8 Interaction of diarrhoeagenic *E.coli* with the epithelial cells of the gut (Modified from Maciver, S.K 2002 and Kaper *et al* 2004).

A. ETEC adhere to small bowel enterocytes and induce watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins.

B. EPEC adhere to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion (pedestal formation). Cytoskeletal derangements are accompanied by an inflammatory response and diarrhoea.

C. EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments.

D. EHEC also induce the attaching and effacing lesion, but in the colon. The distinguishing feature of EHEC is the production of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications.

E. EAEC adheres to small and large bowel epithelia forming a thick biofilm matrix and elaborates secretory enterotoxins and cytotoxins.

F. DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections. (DAF: decay-accelerating factor).

1.2.1.6 Uropathogenic *E. coli* (UPEC)

E. coli is the most common cause of urinary tract infections (UTI's) worldwide, accounting for approximately 95% of community acquired infections and 50% of hospital acquired infections (Foxman & Brown, 2003; Kucheria *et al.*, 2005). As previously mentioned, UPEC isolates are classified as extra intestinal pathogenic *E. coli* (ExPEC), a genetically diverse group of *E. coli* strains that can cause sepsis, UTIs and neonatal meningitis. Interestingly, ExPEC are believed to originate in the gut where they do not appear to cause any form of diarrhoeal disease (Dhakal *et al.*, 2008).

The urinary tract is normally a sterile environment kept free of microbes by antimicrobial peptides, flowing urine and effector immune cells. However, UPEC have developed an array of diverse virulence factors to aid their survival in this harsh environment. These include the expression of P (pap) fimbriae, afimbrial adhesins, haemolysin, secreted toxins, and siderophores like enterobactin and aerobactin which sequester iron away from host cells (Bower *et al.*, 2005; Dhakal *et al.*, 2008; Kaper, 2004). UPEC strains possess large and small pathogenicity islands containing sets of genes that are not found in the chromosome of other *E. coli* strains (Kaper, 2004). Infection begins with the colonisation of the bowel with a UPEC strain that can spread to the periurethral area and ascend the urethra to the bladder (Kaper, 2004). Studies using mouse cystitis model systems have shown that UPEC become localised within acidic membrane-bound vacuoles that have many characteristics of late endosomes and lysosomes (Anderson *et al.*, 2003; Eto *et al.*, 2006; Mulvey *et al.*, 1998; Mulvey *et al.*, 2001). Entry of UPEC into host bladder epithelial cells benefit the microbes, providing them with a niche in which to multiply and persist (Dhakal *et al.*, 2008).

Type 1 fimbriae are a major facilitator of host cell invasion by UPEC. The distal part of the tip of Type 1 fimbriae contains the mannose-binding adhesin FimH which can attach to mannose moieties of the uroplakin receptors that coat transitional epithelial cells (Jones *et al.*, 1995; Mulvey *et al.*, 1998; Sauer *et al.*, 2000). FimH-mediated bacterial invasion of bladder cells requires localized rearrangements of the actin cytoskeleton resulting in the engulfment of the adherent bacteria. These internalised

bacteria may persist for long periods, providing a source for recurrent UTIs (Dhakal *et al.*, 2008).

Type 1 fimbriae are continually expressed in cystitis and the infection is confined to the bladder. However, in strains that cause infection of the kidneys (pyelonephritis) the invertible element that controls type 1 fimbriae expression turns to the 'off' position and type 1 fimbriae are no longer expressed. (Gunther *et al.*, 2001). It has been suggested that this allows the bacteria to attach, using P fimbriae, to digalactoside receptors that are expressed on the kidney epithelium (Kaper, 2004; Korhonen *et al.*, 1986). P fimbriae can interact with UPEC-associated cytotoxic factors like α-haemolysin to facilitate bacterial invasion and dissemination within host tissues (Dhakal *et al.*, 2008; Mulvey, 2002). Haemolysin can disrupt tight junctions, modulate host signalling resulting in increased production of IL-6 and IL-8 and induce host cell death (Kaper, 2004; Uhlen *et al.*, 2000).

1.2.1.7 Neonatal meningitis *E. coli* (NMEC)

NMEC is the most common cause of Gram negative neonatal meningitis, with a case fatality rate of 15–40% and severe neurological defects in the majority of the survivors (Dawson *et al.*, 1999; Kaper, 2004). NMEC are classified as extraintestinal pathogenic *E. coli* (ExPEC) and 80% of these strains are of the K1 capsule type (Kaper, 2004). These bacteria translocate from the blood to the central nervous system and levels of bacteraemia correlate with the development of meningitis (Kaper, 2004). Studies have shown that K1 strains use S fimbriae to bind to the surfaces of brain microvascular endothelium in neonatal rats (Parkkinen *et al.*, 1988) and invasion is dependent on the outer membrane protein OmpA. This protein binds to the antigenic determinant GlcNAcβ1-4GlcNAc of the brain microvascular endothelial cell receptor glycoprotein (Prasadarao *et al.*, 1996).

1.3 Adherent-invasive *Escherichia coli* (AIEC) and Crohn's disease

As stated, *E. coli* is increasingly implicated in the pathogenesis of CD (see section 1.1.6.4). *E. coli* DNA has been detected in 80% of microdissected granulomas of CD patients indicating a possible role for *E. coli* in CD lesions (Bringer *et al.*, 2011; Ryan *et al.*, 2004). Previous analysis of bacterial flora associated with early and chronic ileal lesions of CD patients showed that the ileal mucosa of 36.7% of CD patients is abnormally colonized *E. coli* strains compared to only 5-10% of control patients (Darfeuille-Michaud, 2002; Darfeuille-Michaud, 1998; Martin *et al.*, 2004). Similar results showing higher numbers of CD-associated *E. coli* in CD patients compared to asymptomatic controls have been observed in several independent studies from the United Kingdom (Martin *et al.*, 2004), Ireland (Ryan *et al.*, 2004) and America (Dogan *et al.*, 2012). In a study to assess the predominance of *E. coli* strains associated with the ileal mucosa of CD patients, *E. coli* was recovered from 65% of chronic lesions (resected ileum) and from 100% of the biopsies of early lesions (Darfeuille-Michaud, 1998). *E. coli* counts from the rectal mucosa were also observed to be higher in active CD and UC rather than inactive and healthy controls (Mylonaki *et al.*, 2005).

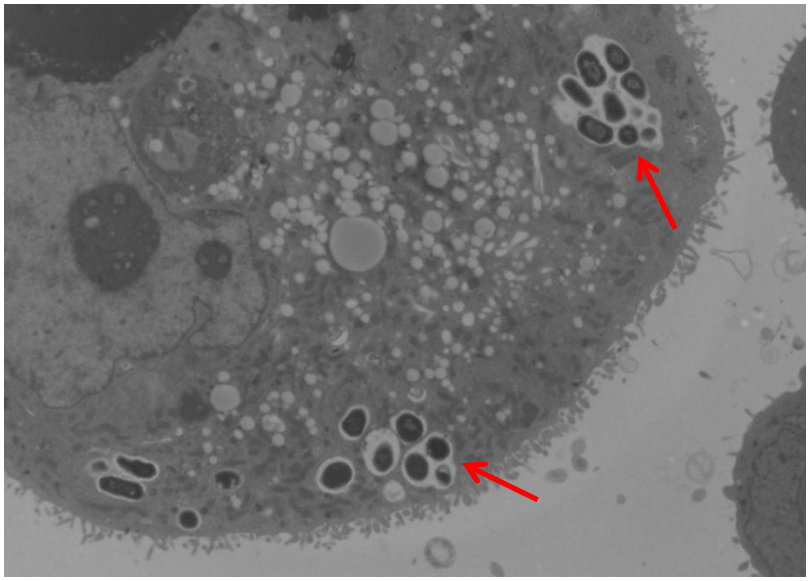
Most of these CD-associated *E. coli* strains can adhere to cultured intestinal epithelial cells such as differentiated Caco-2 cells, a property that could allow them to colonise the intestinal mucosa (Darfeuille-Michaud, 1998). Subsequent studies of the interaction between these strains and cultured human epithelial cells also revealed an invasive phenotype (Boudeau *et al.*, 1999). Furthermore, independent studies have shown that invasive *E. coli* can be found in 29%-36% of CD patients compared to 3%-9% in healthy controls (Martin, 2004). Such studies have led to a new category of *E. coli* designated adherent-invasive *Escherichia coli* (AIEC) (Darfeuille-Michaud, 2002). The high levels of AIEC colonizing the intestinal mucosa of patients with CD suggests that it may play an important role in the etiopathogenesis of CD (Caprilli, 2008). However, it is important to note that AIEC strains do not represent a specific pathogen exclusively found in CD because of their presence in a few ileal or colonic specimens from healthy individuals suggesting that AIEC strains may belong to a transient normal microbiota (Darfeuille-Michaud, 2004).

Most of the experimental data on the AIEC phenotype is based on gene deletion studies with *E. coli* LF82 (serotype O83:H1), isolated in France from an ileal biopsy of a patient with CD (Darfeuille-Michaud, 1998). Other CD-associated *E. coli* strains with similar properties have been isolated suggesting these observations are generalizable (Wine *et al.*, 2011). However, MLST analysis suggests that *E. coli* isolated from CD patients did not evolve from a unique ancestral background and not all of these phenotypically-defined strains may share all of the same proposed virulence genes (Eaves-Pyles, 2008; Sepehri *et al.*, 2009; Wine *et al.*, 2011). The current criteria for inclusion into the AIEC group are (i) the ability to colonies the intestinal mucosa by adhering to and invading intestinal epithelial cells with a macropinocytosis-like process of entry dependent on actin microfilaments and microtubule recruitment, (ii) the ability to survive and to replicate in large vacuoles within macrophages and (iii) the ability to induce the release of high amounts of pro-inflammatory cytokines such as TNF-α and IL1-β (Boudeau *et al.*, 1999; Boudeau *et al.*, 2001; Darfeuille-Michaud, 2002; Darfeuille-Michaud, 2004; Martin *et al.*, 2004).

1.3.1 AIEC and the Epithelial Barrier

Intestinal epithelial cells not only act as a physical barrier in preventing enteric bacteria from entering and interacting with immune cells in the lamina propria but these cells also act as innate immune sensors for pathogens, as well as commensals (Kagnoff & Eckmann, 1997). Bacterial adhesion to intestinal epithelial cells is the first step in the pathogenicity of many bacteria involved in infectious diseases of the gut. Adhesion limits bacterial clearance from the intestine and enables the bacteria to colonise the gut (Eaves-Pyles, 2008). Interestingly, it was shown using PCR that the AIEC strain LF82 does not possess any of the known virulence factors normally associated with pathogenic *E. coli* involved in gastrointestinal outbreaks (Darfeuille-Michaud, 1998; Martin *et al.*, 2004), for example the *ipaB, ipaC* and *ipaD* plasmid genes encoding the invasion phenotype of EIEC, the *eae* gene encoding the intimin of EPEC, or the *tia* gene encoding a 25-kilodalton outer membrane protein involved in ETEC invasiveness (Darfeuille-Michaud, 2004).

A



B

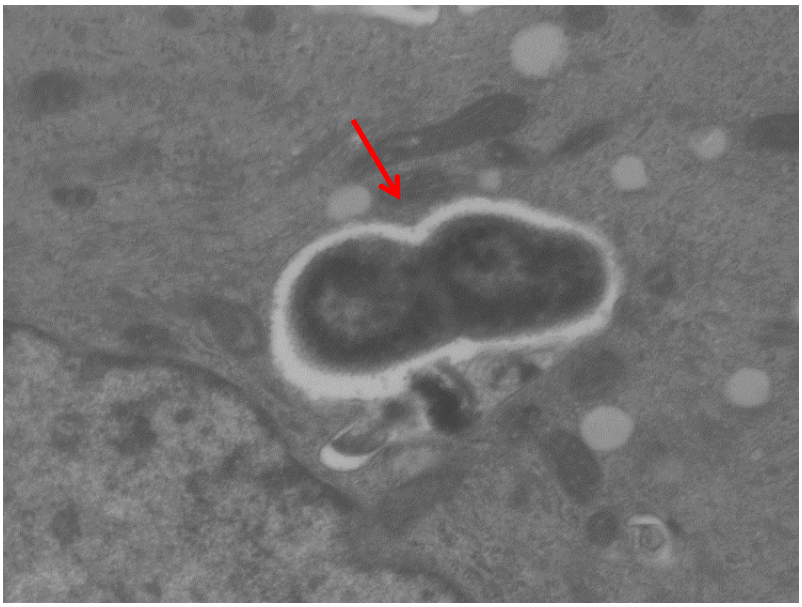


Figure 1.9 Transmission electron micrographs of C2Bbe1 epithelial cells infected with AIEC strain HM605. A) A section of the cell monolayer showing numerous intracellular bacteria after a 4h infection period. B) High magnification showing invasion of bacteria into epithelial cell. Septum formation on the bacteria indicates that the strain HM605 can survive and replicate inside C2Bbe1 epithelial cells. Magnification: A, X4,000; B, X30,000.

1.3.1.1 Type 1 pili and flagella mediated adhesion/invasion to epithelial cells.

Type 1 fimbriae-mediated adherence is involved in LF82 invasion of intestinal epithelial cells (Boudeau *et al.*, 2001). Type 1 fimbriae are the most common filamentous bacterial appendages of many Gram-negative bacteria such as *E. coli* and they promote bacterial adhesion to various types of eukaryotic cells. By using Tn*phoA* insertion mutagenesis, non-invasive mutants of LF82 were identified and subsequently were shown to have the transposon inserted into the type 1 fimbriae-encoding operon. Such mutations showed a decrease in adherence suggesting the involvement of type 1 fimbriae in adherence of AIEC (Boudeau *et al.*, 2001). The type 1 fimbriae-encoding operon is composed of nine genes and the fimbriae itself consists of the major subunit FimA and the three minor subunits FimF, FimG and FimH (Jones *et al.*, 1995; Klemm & Christiansen, 1990). As stated, FimH binds to terminally located D-mannose moieties on cell-bound and secreted glycoproteins that coat transitional epithelial cells allowing bacterial adhesion (Boudeau *et al.*, 2001). It is well documented that the adhesin FimH is involved in the virulence of UPEC, mediating invasion of host bladder epithelial cells via initiation of host cell signalling cascades (see section 1.2.1.6). Multiple alignment of the predicted amino acid sequence of FimH for LF82 and K-12 strains showed that variations occurred in the predicted LF82 FimH sequence indicating point amino acid substitutions along the entire length of the sequence (Barnich & Darfeuille-Michaud, 2007b; Boudeau *et al.*, 2001). Interestingly, LF82 *fimH* sequence showed strong homology with invasive *E. coli* strains, mainly NMEC strain IHE3034 and APEC strain MT78 (Boudeau *et al.*, 2001).

In LF82, type 1 fimbriae trigger the formation of membrane extensions at the sites of intimate contact between the entering bacteria and the epithelial cells. These membrane extensions surround the bacteria and allow for uptake into the epithelial cell. Membrane extensions are not observed when epithelial cells are infected with LF82 *fimF* and *fimI* mutant cells that are unable to produce Type 1 fimbriae and consequently invasion is reduced (Boudeau *et al.*, 2001). Therefore, LF82 adhesion to, and invasion of, epithelial cells depends on type 1 fimbriae triggering these membrane extensions in epithelial cells. Interestingly the expression of the Type 1 fimbriae from LF82 in another, non-invasive strain of *E. coli*, did not induce invasiveness highlighting that there are other genes in LF82 that are required for

invasion (Boudeau *et al.*, 2001). Interestingly, dose dependent degradation of type 1 pili in the presence of the protease meprin impairs the ability of LF82 to bind to mannosylated host receptors on epithelial T84 cells and leads to decreased production of the pro-inflammatory cytokine IL-8. Proteases can act as host defence mechanisms to counteract bacterial colonization. Meprin is abundantly expressed in intestinal epithelial cells, however, decreased levels of the meprin are observed in CD patients and consequently it has been speculated that this may contribute to increased AIEC colonization (Vazeille *et al.*, 2011). It must be noted that the studies above focused on LF82, other studies have shown that colonic AIEC isolates expressing *fimH* also bind to intestinal epithelial cells but in a mannose independent manner e.g. AIEC HM605 (Martin *et al.*, 2004; Simpson *et al.*, 2006)

Flagella have also been shown to play a direct role in adherence and an undefined role in invasion of epithelial cells by LF82 (Barnich *et al.*, 2003; Eaves-Pyles, 2008). The transcriptional activator of flagellar biogenesis, $FlhD_2C_2$ and the sigma factor FliA have been shown to be involved in the coordination of flagellar and type 1 fimbriae synthesis. If *fliA* is deleted in LF82, type 1 fimbriae formation and adherence to epithelial cells is dramatically decreased due to the intracellular concentration of the cyclic-di-GMP secondary messenger (Claret, 2007). The loss of type 1 fimbriae in the *fliA* was attributed to the decreased expression of the FliA dependent *yhjH* gene in the LF82 *ΔfliA* mutant (Claret, 2007). YhjH is an EAL domain phosphodiesterase involved in degradation of the bacterial second messenger c-di-GMP and in LF82 it was reported that lower c-di- GMP levels seem to stimulate type 1 fimbriae synthesis (Claret, 2007). This indicates LF82 FliA is a key regulatory component linking flagellar and type 1 fimbriae synthesis and furthermore LF82 FliA effect on type 1 pili is appears to mediated via a c-di-GMP dependent pathway (Claret, 2007). AIEC flagellin has also been shown to induce the release of the pro-inflammatory cytokine IL-8 (Subramanian, 2008).

The non-motile aflagellar *fliC* mutant also shows a drastic down-regulation of type 1 fimbriae synthesis and a decrease in the adhesive and invasive abilities of LF82 (Claret, 2007). Such studies demonstrate that flagellar motility and other factors (e.g. Type 1 fimbriae) are co-regulated in LF82. Other adhesins such as the lipoprotein NlpI have been reported to play a role in adherence and invasion of LF82 (Barnich *et*

al., 2004). Surface-exposed lipoproteins can play a role as adhesins and NlpI is reported to be targeted to the outer membrane in the *E. coli* (Barnich *et al.*, 2004). An *nlpI*-negative isogenic mutant was shown to be reduced in its ability to adhere to and invade Intestine-407 cells. Although both type 1 fimbriae and flagella were not expressed in this mutant the adherence/invasion phenotype was shown to be independent of both type 1 fimbriae and flagella.

1.3.1.2 Type 1 fimbriae and Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM-6) expression

The high prevalence of AIEC strains associated with the ileal mucosa observed in CD patients suggests that the abnormal expression of a specific host receptor on host epithelial cells may be recognized by bacterial lectin-like surface adhesins in a genetically predisposed host (Barnich *et al.*, 2007). Consequently, it was shown that ileal AIEC adhesion to epithelial cells depends on type 1 fimbriae expression on the bacterial cell surface and the recognition of the glycoprotein carcinoembryonic antigen–related cell adhesion molecule 6 (CEACAM-6) on the apical surface of ileal epithelial cells by type 1 fimbriae (see fig 1.10) (Barnich & Darfeuille-Michaud, 2007a; Barnich & Darfeuille-Michaud, 2010). A number of glycosylated receptors for type 1 fimbriae have been documented, however western blot analysis of total protein extract from ileal specimens of 9 CD patients and 9 healthy controls showed an abnormally strong expression of CEACAM-6 on the brush border of enterocytes in the ileal mucosa of CD patients in both active and inactive areas compared to controls (Barnich *et al.*, 2007). No up-regulation of CEACAM-6 expression was observed in the colonic mucosa indicating that the up-regulation of CEACAM-6 expression is specific to the ileal segment of the intestine of CD patients (Barnich *et al.*, 2007). Interestingly, *in vitro* studies show an increase in epithelial CEACAM-6 expression after infection with AIEC strain LF82 and after stimulation with IFN-γ or TNF-α (Barnich *et al.*, 2007; Barnich & Darfeuille-Michaud, 2010). This suggests that inflammatory conditions and/or infection with AIEC can induce CEACAM-6 expression in intestinal epithelial cells and LF82 may, therefore, promote its own colonisation in CD patients. Similarly, LF82 adhesion to intestinal epithelial cells was significantly increased when the expression of CEACAM-6 was induced after IFN-γ stimulation highlighting that the adhesion of AIEC increases with CEACAM-6 expression (Barnich *et al.*, 2007).

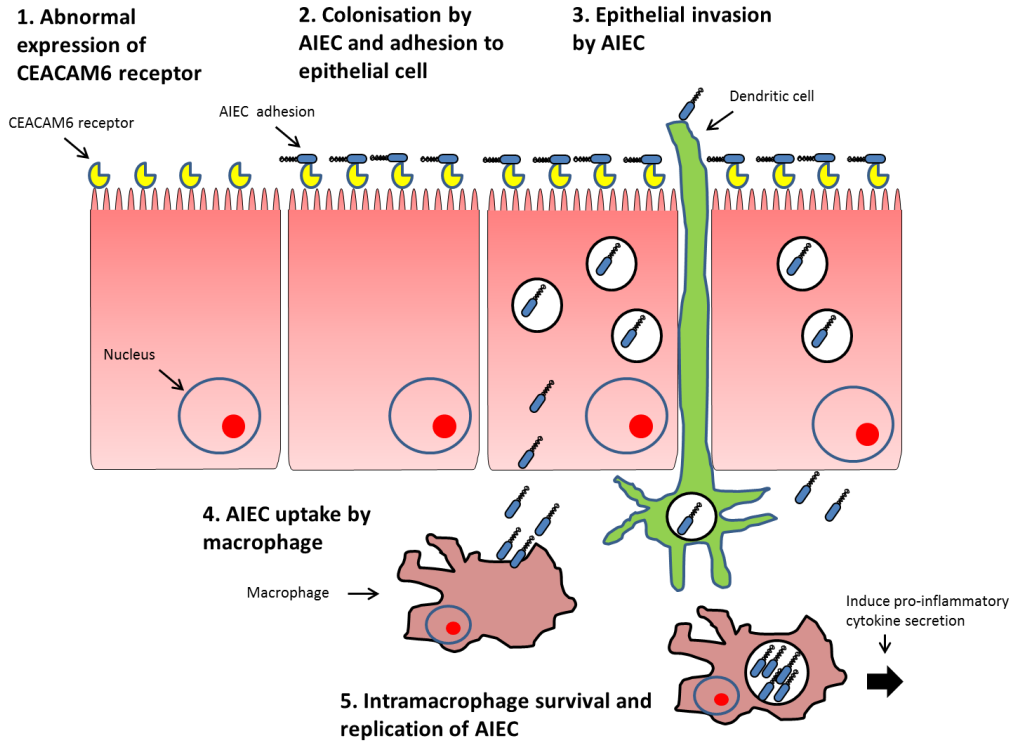


Figure 1.10 Stages of AIEC colonization in the ileal mucosa.

(1) The abnormal expression of CEACAM-6 in ileal results AIEC LF82 binding to CEACAM-6 allowing for (2) AIEC colonization, adhesion and (3) epithelial invasion, which allow the bacteria to cross the mucosal barrier. (4) AIEC bacteria are taken up by macrophage and can (5) survive and replicate within infected macrophages in the lamina propria, and induce pro-inflammatory secretion

In vivo, AIEC expressing type 1 fimbriae bind to CEACAM-6 allowing AIEC to colonize the intestinal mucosa of transgenic mice expressing human CEACAM-6 and also induce gut inflammation (Carvalho *et al.*, 2009). Absence of colonization and inflammation was observed when the non-piliated mutant of LF82, which is unable to bind CEACAM-6, was orally administered to transgenic mice (Carvalho *et al.*, 2009). This indicates that the adhesion of LF82 to the ileal mucosa via type 1 fimbriae binding to CEACAM-6 is essential for LF82 adhesion and for the development of inflammation (see Fig. 1.10). In a recent study, infection of CEABAC10 transgenic mice expressing human CEACAM-6 with LF82, but not with non-pathogenic *E. coli*, led to a significant 3-fold increase in intestinal permeability and disruption of mucosal integrity, in a type 1 fimbriae-dependent manner (Denizot *et al.*, 2011). This is consistent with the abnormal expression of the pore-forming tight junction protein claudin-2 at the plasma membrane of intestinal epithelial cells observed in AIEC-infected CEABAC10 mice (Denizot *et al.*, 2011). These findings suggest that LF82 type 1 fimbriae-mediated interaction with CEACAM-6 may also disrupt intestinal barrier integrity.

1.3.1.3 AIEC and M-cells: A potential entry point.

Clinical observations suggest that the sites of initial inflammation in ileal CD are the lymphoid follicles as erosions can be seen at the specialized follicle associated epithelium (FAE), which lines Peyer's patches (PPs) (Chassaing *et al.*, 2011; Fujimura *et al.*, 1996). Epithelial cells emerging from the follicle-associated crypts migrate as a dome is formed by the underlying lymphoid follicle, thus they are often referred to as dome epithelial cells (Kraehenbuhl & Neutra, 2000). FAE contain specialized membranous/microfold cells (M cells) that are involved in the immunological sampling of microorganisms and foreign antigens (Kraehenbuhl & Neutra, 2000). M cells facilitate the adherence and uptake of microorganisms and microbial antigens on their apical microfold membranes and allow for presentation to lymphoid or antigen-presenting cells in the sub-epithelial tissue (Knight *et al.*, 2008; Kraehenbuhl & Neutra, 2000). They account for approximately 3% of the cells in the dome epithelium that overlies PPs in the distal small intestine and the lymphoid follicles in the colon (Knight *et al.*, 2008). A number of pathogens exploit the features of M cells that are intended to promote uptake for the purpose of

immunological sampling and use them to cross the intestinal barrier, for example invasive pathogens such as *Shigella* and *Salmonella* are unable to invade via normal colon cells but enter via M cells (Knight *et al.*, 2008; Sansonetti & Phalipon, 1999). *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* also cross the intestinal epithelial barrier through M cells of the FAE (Marra & Isberg, 1997) and EPEC translocate across an *in vitro* M cell model under the regulation of a type III secretion system (Martinez-Argudo *et al.*, 2007). M cells have a number of features that allow for such exploitation: a poorly organized brush border, the lack of goblet cells and as a result a lack of mucus covering the intestinal epithelial cells facilitating contact between the bacteria and the M cells (Knight *et al.*, 2008; Neutra *et al.*, 1999). As other invasive pathogens utilise M cells as an entry point into epithelium, it is likely that AIEC may also enter in this manner. Evidence for this comes from M cells cultured *in vitro* (Roberts et al., 2010). An interesting observation is that the peaks of greatest cases of CD occur between 15-25 years of age, this correlates with the peak number of PP (Van Kruiningen *et al.*, 1997). This correlation suggests that CD may develop as an inflammatory process specifically targeting M cells. It has been recently reported that a protein located on the apical plasma membrane of M cells among enterocytes interacts with type 1 fimbriae on bacteria (Hase *et al.*, 2009). Glycoprotein 2 (GP2) recognized the FimH adhesin of type 1 fimbriae on S*almonella enterica* serovar Typhimurium and *E. coli* and suggests that entry into M cells could occur via the recognition of FimH by GP2 (Hase *et al.*, 2009). Interestingly, in S*almonella enterica* serovar Typhimurium appendages known as long polar fimbriae (LPF) are responsible for specific adherence to M cells of the murine FAE (Baumler *et al.*, 1996). Moreover, in addition to type 1 fimbriae, the AIEC strain LF82 also expresses long polar fimbriae (LPF), encoded by the *lpf* operon (Chassaing *et al.*, 2011). A recent study reported that the LPF of LF82 interact with murine and human PPs and ultimately allow translocation across M cells (Chassaing *et al.*, 2011). An isogenic mutant, *ΔlpfA,* in LF82 (which still have type 1 fimbriae) was reduced in its ability to interact with murine and human isolated PPs and was also impaired in its ability to translocate across M-like cell monolayers and to interact with murine M cells *in vivo* (Chassaing *et al.*, 2011). These results indicate that LPF of LF82 play a key role in targeting M cells which could allow subsequent entry into lymphoid tissue. As stated, type 1 fimbriae (FimH) binds to GP2 therefore it is likely that LPF is not binding to GP2 directly but is binding via a

mannose-independent manner (associated with GP2 positive cells). LPF expression is not necessary for LF82 binding to polarized enterocytes, this binding is associated solely with CEACAM-6 and type 1 fimbriae (Chassaing *et al.*, 2011; Strober, 2011).

Interestingly, a high prevalence of putatively functional *lpfA*-positive AIEC strains was observed in CD patients compared to control patients (21.8% and 3.5% respectively) and most of the *E. coli* strains associated with the ileal mucosa of controls were non-AIEC and did not harbor the *lpfA* gene (Chassaing *et al.*, 2011). Since the initial lesions in CD appear to occur at PPs CD-associated AIEC expressing LPF could use PPs as an open gate promoting invasion. It was also observed that *NOD2*-deficient mice expressed increased amounts of GP2 on M cells and consequently had increased numbers of LPF+ AIEC bacteria; however this did not induce colitis in the mice (Chassaing *et al.*, 2011). It should be noted that this does not imply that LPF binds to GP2 as a previous study has shown that bile salts increase the expression of LPF on AIEC (Chassaing *et al.*, 2012).

1.3.1.4 Outer membrane vesicles (OMVs) contribute to AIEC invasion

Outer membrane vesicles (OMVs) are a means by which bacteria interact with prokaryotic and eukaryotic cells in their environment (Kuehn & Kesty, 2005). OMVs arise from the surfaces of many Gram-negative bacteria and consist of outer membrane and entrapped periplasmic components (Rolhion *et al.*, 2005). In pathogenic bacteria OMVs contain adhesins, toxins, and other virulence factors for intercellular transport and they directly mediate bacterial binding and invasion as well as modulating the host immune response (OMVs have been shown to be pro-inflammatory) (Kuehn & Kesty, 2005; Subramanian, 2008). OMVs therefore act as potent virulence factors in the host-bacteria interaction. For example, OMVs are associated with the IpaB, IpaC and IpaD virulence factors in *S. flexneri* (Kadurugamuwa & Beveridge, 1998) as well as delivery of the LT toxin in ETEC (Wai *et al.*, 1995).

In AIEC, OMVs may act as a potential virulence factor as it has been reported that deletion of *yfgL* gene leads to a strong decrease in OMVs release and consequently a reduction in the invasive ability of LF82 into Intestine-407 epithelial cells (Rolhion

et al., 2005). The *yfgL* gene encodes the YfgL lipoprotein involved in the syntheses and/or degradation of peptidoglycan (Eggert *et al.*, 2001). The deletion of *yfgL* gene in LF82 was also shown to affect the release of the integral outer membrane proteins OmpA and OmpC in the culture supernatant by decreasing the release of OMVs compared to wildtype (Eggert *et al.*, 2001). The protein OmpA has been previously reported act as a virulence factor playing many important roles in adhesion, invasion and persistence of *E. coli*. For example, in NMEC OmpA is required for invasion into brain microvascular endothelial cells by interacting with the endothelial cell glycoprotein Ecgp96 (Prasadarao, 2002; Rolhion *et al.*, 2010). In intestinal epithelial cells a homologue of Ecgp96, known as the endoplasmic reticulum (ER)-localised stress response chaperone Gp96 can be found (Cabanes *et al.*, 2005; Rolhion *et al.*, 2010). Interestingly, it has been recently reported that OmpA at the surface of LF82 OMVs interacts with epithelial Gp96 allowing the adhesion and invasion of LF82 to intestinal epithelial cells (Rolhion *et al.*, 2010). Deletion of the *ompA* gene markedly reduced LF82 invasion of Intestine-407 epithelial cells and exhibited a loss of fusion of OMVs with the host cell. Gp96 is overexpressed at the apical plasma membrane of ileal epithelial cells of patients with CD (Rolhion *et al.*, 2010). This suggests that in CD patients, AIEC may take advantage of this Gp96 overexpression to allow delivery into host cells of virulence factors that contribute to the invasion process via OMVs (Rolhion *et al.*, 2011). Similarly, deletion of *ompC* in LF82 significantly reduced the adhesion and invasion levels of LF82 into Intestine-407 epithelial cells (Rolhion *et al.*, 2007). This defect was shown to be independent of type 1 fimbriae and flagella. However, under conditions of high osmolarity, similar to that in the GIT, increased expression of OmpC in LF82 and activation of the RpoE (σ^E) regulatory pathway which responds to envelope stress was observed (Rolhion *et al.*, 2007). Activation of the RpoE pathway can regulate the expression of several virulence factors promoting AIEC adhesion and invasion into intestinal epithelial cells as well as biofilm formation (Chassaing *et al.*, 2012; Rolhion *et al.*, 2007).

Table 1.2 Key virulence factors associated with epithelial cell invasion in AIEC LF82

Virulence factor	Function	References
Type 1 fimbriae	Induce membrane extensions	(Barnich *et al.*, 2003; Boudeau *et al.*, 2001)
Flagella	Bacterial mobility and down regulation of Type 1 fimbriae	(Barnich *et al.*, 2003; Eaves-Pyles, 2008)
Outer membrane vesicles (OMVs)	Delivery of bacterial effector proteins to host cells	(Rolhion *et al.*, 2005)
Long polar fimbriae (LPF)	Interact with Peyer's patches and M-cells	(Chassaing *et al.*, 2011)
OmpA	Interacts with Gp96 on host membrane	(Rolhion *et al.*, 2010; Rolhion *et al.*, 2011)
OmpC	Regulates the expression of several virulence factors via RpoE pathway	(Rolhion *et al.*, 2007)
NlpI	Adherence/invasion of epithelial	(Barnich *et al.*, 2004)

1.3.2 Replication in macrophages.

In the intestine, macrophages are located underneath the intestinal epithelium, with the lamina propria containing the largest number of macrophages in the body (Barnich *et al.*, 2004). Macrophages act as the first line of defence, eliminating unwanted microorganisms by engulfing them into phagosomes, which rapidly develop into bactericidal phagolysosomes after fusion with lysosomes (Barnich *et al.*, 2004). Invasive bacteria such as *M. tuberculosis*, *Legionella pneumophila* and *Listeria monocytogenes* have developed various methods to counteract and/or disrupt these mechanisms, which, when successful, enable them to survive, replicate and, in some cases, lyse macrophages (see fig.1.11) (Finlay & Cossart, 1997; Flannagan *et al.*, 2009; Glasser *et al.*, 2001). Once through the epithelial barrier, AIEC are phagocytised by macrophages. Evidence for this is highlighted by the isolation of *E. coli* in macrophages localised in the lamina propria, in granulomas and also within mesenteric lymph nodes of CD patients (Lodes *et al.*, 2004). A number of studies

have shown that AIEC strains isolated from ileal and colonic CD patients are capable of surviving and replicating within J774A.1 murine macrophages and primary human monocyte derived macrophages (Darfeuille-Michaud *et al.*, 2004; Glasser *et al.*, 2001; Meconi *et al.*, 2007; Mpofu *et al.*, 2007; Subramanian, 2008).

1.3.2.1 AIEC replicates in mature phagolysosomes

Upon infection, LF82 is engulfed by macrophage within phagosomes that rapidly evolve and mature into organelles termed phagolysosomes (Bringer *et al.*, 2006). As phagosomes mature they acidify and interact with the endosomal network and/or the biosynthetic pathway (Bringer *et al.*, 2006; Desjardins, 1995). After infection LF82-containing phagosomes acquire early endosome antigen 1 EEA1. This protein is associated with early endosome development (Desjardins, 1995). This allows for the late endosome stage of maturation, characterised by the acquisition of Rab7 GTPase were the phagosome continues to acidify (Bringer *et al.*, 2006). In pathogenic bacteria such as *Mycobacterium tuberculosis* and *Mycobacterium* avium, maturation of the phagosome is blocked by inhibiting stages controlled by Rab5 and Rab7 resulting in the retention of Rab7 GTPase on the phagosome. This allows the bacteria to replicate within an immature phagosome (Clemens & Horwitz, 1995). However, in LF82-infected macrophages, the Rab7 GTPase was not retained on the phagosome, indicative of phagosome maturation into a phagolysosome (Bringer *et al.*, 2006; Clemens & Horwitz, 1995). Following this maturation, peripheral transmembrane glycoproteins (Lamps) are observed on the LF82-containing phagosome and intraluminal concentrations of the degradative protease cathepsin D are increased. Therefore, LF82-containing phagosomes mature into active phagolysosomes where they are exposed to low pH and also to degradative proteases (Bringer *et al.*, 2006).

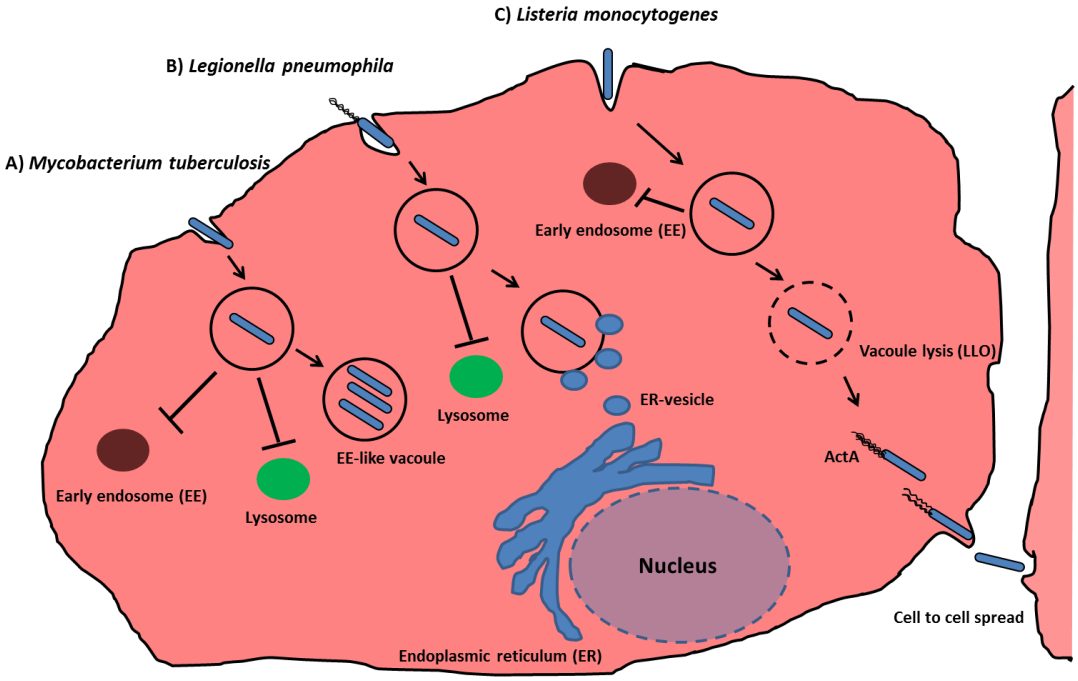


Figure 1.11 Strategies used by three intracellular bacterial pathogens to modulate phagosome maturation for survival inside the macrophage.

A) *M. tuberculosis*. After internalization, the bacterium uses a number of effector molecules, including the lipids phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM), and the phosphatidylinositol-3-phosphate (PI-3-P) phosphatase SapM to stop phagosome maturation at the early stage.

B) *L. pneumophila*. This bacterium impairs fusion of the Legionella-containing vacuole with endolysosomal compartments and following this promotes fusion with endoplasmic reticulum (ER)-derived membranes.

C) *L. monocytogenes*. This pathogen evades phagolysosomal fusion after internalization by escaping the phagosome through secretion of listeriolysin O (LLO) and two phospholipases, PlcA and PlcB. Once in the cytoplasm, *L. monocytogenes* can undergo replication and become motile by manipulating host cell actin inducing 'tails' generated by the effector ActA.

Interestingly, when macrophages were treated with vacuolar pH-neutralizing agents such as chloroquine and ammonium chloride the intracellular replication of LF82 was inhibited suggesting that an acidic pH is required for LF82 replication and may switch on expression of virulence genes which allow these bacteria to replicate (Bringer *et al.*, 2006). Moreover, it has been previously reported that the stress protein HtrA plays an essential role in the adaptation of intracellular AIEC LF82 bacteria to acidic pH conditions (Bringer *et al.*, 2005). Deletion of the *htrA* gene in LF82 induced increased sensitivity of the isogenic mutant to oxidative stress caused by hydrogen peroxide and low growth in an acid and nutrient-poor medium partly reproducing the microenvironment of the phagosome (Bringer *et al.*, 2005). Furthermore, *in vitro* experiments showed that the *htrA* gene was 38-fold up-regulated in LF82 intramacrophagic bacteria. These studies indicate the importance of phagosomal acidification for intramacrophagic survival and/or replication of LF82 (Bringer *et al.*, 2005). Similarly, *htrA* has also been shown to be important for the intracellular replication of *Salmonella* (Baumler *et al.*, 1994; Eriksson *et al.*, 2003), *Legionella pneumophila* (Pedersen *et al.*, 2001) and *Brucella abortus* (Elzer *et al.*, 1996) in macrophage cells.

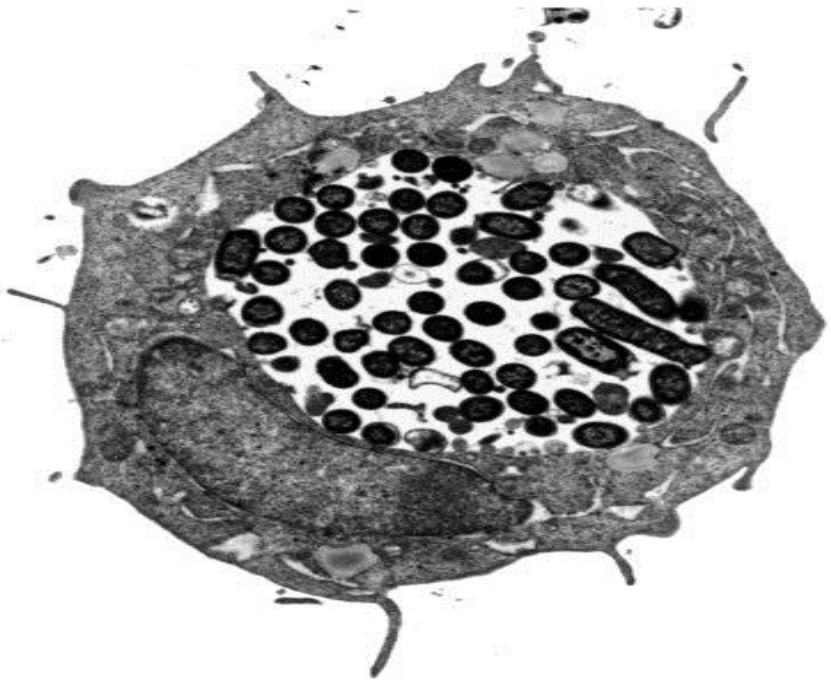


Figure 1.12: Transmission electron micrograph of LF82 inside J774-A1 macrophages. Large numbers of intracellular AIEC LF82 in a large vacuole 48 hours after infection (From Caprilli R *et al* 2010).

1.3.2.2 The periplasmic oxidoreductase DsbA and the RNA binding protein Hfq are required for intramacrophage replication of AIEC strain LF82.

Many strategies for intramacrophage survival involve proteins that are exported from the cytoplasm to either the periplasm or the outer membrane or are secreted out of the cell (Bringer *et al.*, 2007; Bruggemann *et al.*, 2006; Celli, 2006). Many proteins, especially membrane and exported proteins are stabilized by intramolecular disulfide bridges between cysteine residues which are essential for the correct functioning of proteins (Bringer *et al.*, 2007). In *E. coli*, the Dsb proteins are responsible for the formation of disulfide bonds (Heras *et al.*, 2009). DsbA is a 21-kDa periplasmic protein and is responsible for the introduction of disulfide bonds to newly synthesised proteins (Bringer *et al.*, 2007; Heras *et al.*, 2009). The activity of DsbA is maintained by the function of the inner membrane protein DsbB, which oxidizes DsbA (Bringer *et al.*, 2007; Heras *et al.*, 2009).

The role of DsbA in the virulence of pathogenic *E. coli* has been previously reported. It has been shown to be involved in the biogenesis of bacterial cell surface appendages such as flagella (Dailey & Berg, 1993) and fimbriae in EPEC and UPEC (Donnenberg *et al.*, 1997; Jacob-Dubuisson *et al.*, 1994). Similarly, it is crucial in the biogenesis of toxins such as the heat-labile and heat-stable toxins of ETEC (Okamoto *et al.*, 2001). In LF82 it was shown that a Δ*dsbA* mutant was unable to replicate in macrophages (Bringer *et al.*, 2007). In addition, LF82 Δ*dsbA* was affected in the ability to adhere to and invade Intestine-407 epithelial cells, suggesting that DsbA is essential for multiple virulence functions in AIEC strain LF82 (Bringer *et al.*, 2007). Similar to the *htrA* gene, transcription of the *dsbA* gene was up-regulated when LF82 was grown *in vitro* in a medium that partly mimicked conditions encountered in the phagolysosome, suggesting that DsbA is activated under phagolysosomal conditions (Bringer *et al.*, 2007). Therefore, the DsbA oxidoreductase is essential for LF82 to resist killing in the hostile environment of the phagosome. Similarly to the LF82 *dsbA* mutant, a *S. flexneri dsbA* mutant also grow poorly within macrophages (Yu *et al.*, 2001).

It has also been reported that the RNA binding protein, Hfq, is important for LF82 survival and multiplication within macrophages (Simonsen *et al.*, 2011). Hfq is a small RNA-binding protein that functions as a global posttranscriptional regulator of

gene expression by binding to small regulatory RNA molecules (sRNA) and facilitating their interaction with their target mRNA molecules (Ansong *et al.*, 2009; Simonsen *et al.*, 2011). Deletion of *hfq* in LF82 resulted in reduced host cell invasion as well as reduced intracellular survival and replication in cultured macrophages (Simonsen *et al.*, 2011). Moreover, deletion of *hfq* increased sensitivity of LF82 to stress conditions encountered within the phagolysosome, such as low pH, reactive oxygen species and reactive nitrogen species (Ansong *et al.*, 2009). These results demonstrate that LF82 Hfq may play an important role in promoting bacterial adaptation to the multiple stress conditions encountered in the mature phagolysosome.

1.3.2.3 Defects in autophagy enhance intracellular replication of AIEC strain LF82

As previously mentioned, CD associated polymorphisms in *NOD2, ATG16L1* and *IRGM* affect bacterial autophagy (see table 1.0). Defects in autophagy lead to abnormal immune responses to intracellular pathogens and may contribute to the pathogenesis of CD (Lapaquette *et al.*, 2012b). Moreover, dysfunction in autophagy leads to persistent infection of intracellular pathogens such as *S. enterica* serovar Typhimurium, *Streptococcus pyogenes*, and *M. tuberculosis* (Birmingham *et al.*, 2006; Nakagawa *et al.*, 2004; Singh *et al.*, 2006). It was recently reported that LF82 induced autophagy in mouse embryonic fibroblasts (MEFs) and autophagy restricted the intracellular replication of LF82. In addition, LF82 were shown to interact with autophagy in a variety of epithelial cell lines (Lapaquette *et al.*, 2012c). Treatment of HeLa cells with rapamycin to induce autophagy significantly reduced the number of intracellular LF82 bacteria in a dose-dependent manner (Lapaquette *et al.*, 2012c). However, intracellular AIEC LF82 was shown to replicate within HeLa cells having impaired ATG16L1 expression. A reduction in ATG16L1 expression allowed for a significant increase in the numbers of intracellular AIEC LF82 bacteria at 1 and 6 h post infection (Lapaquette *et al.*, 2012c).

It is also been recently reported that AIEC LF82 induces recruitment of the autophagic machinery at the site of entry in human monocyte-differentiated THP-1 macrophages (Lapaquette *et al.*, 2012b). Autophagy was analysed by comparing LC3-II turnover in the presence or absence of Bafilomycin A1, a lysosomal inhibitor that blocks autophagosome maturation (Klionsky *et al.*, 2008). A significant increase

in LC3-II accumulation was observed at early time point post infection in Bafilomycin A1-treated THP-1 macrophages in response to infection, indicating stimulation of autophagy by AIEC (Lapaquette *et al.*, 2012b). ATG16L1- and LC3-positive AIEC-containing vacuoles were closely associated with actin rearrangements induced for bacteria engulfment which indicates that the autophagic proteins were recruited at the LF82 entry site. This suggests that invading AIEC LF82 is immediately brought into contact with the autophagy machinery (Caprilli *et al.*, 2010; Lapaquette *et al.*, 2012b). Furthermore, similar to autophagy in epithelial cells, siRNA analyse showed that decreased expression of *ATG16L1*, *IRGM* and also *NOD2* significantly increases the numbers of intramacrophagic LF82 bacteria in THP-1 macrophages (Lapaquette *et al.*, 2012b). Thus, the two autophagy-related proteins ATG16L1 and IRGM as well as NOD2-dependent autophagy control the number of intracellular AIEC LF82 bacteria in human THP-1 macrophages. Interestingly, it was also shown that defective autophagy results in an increased release of the pro-inflammatory cytokines TNF-α and IL-6 by LF82-infected macrophages (Lapaquette *et al.*, 2012b). These findings show that lack of autophagic response resulting from altered *ATG16L1*, *IRGM* or *NOD2* expression favours AIEC LF82 replication inside the macrophage (Lapaquette *et al.*, 2012b).

1.4 Comparative genomics.

To date the genomes of four AIEC strains, LF82 (Miquel *et al.*, 2010), HM605 (Clarke *et al.*, 2011), UM146 (Krause *et al.*, 2010) and NRG857c (Nash *et al.*, 2010) have been published. These genomes, and other comparative genomic studies, suggest that AIEC most closely resemble ExPEC (Martinez-Medina *et al.*, 2009b). Moreover, AIEC strains cluster genetically with ExPEC and share some of their phenotypic traits including the ability to colonize mucosal epithelial cells, invade eukaryotic host cells, and to induce inflammatory responses (Nash *et al.*, 2010). Interestingly, a later study observed that, although AIEC and ExPEC have similar virulence gene profiles and phylogenetic backgrounds, the majority of ExPEC do not behave phenotypically like AIEC (Martinez-Medina *et al.*, 2009d). Phylogenetic analyses of AIEC NRG857c and comparison of this genome with other ExPEC, AIEC LF82, EPEC and commensal *E. coli* facilitated the identification of 35 high-confidence genomic islands and 66 genes unique to *E. coli* displaying the adherent

and invasive phenotype (Nash *et al.*, 2010). The conservation of these 35 genomic islands between NRG8578c and LF82 was high, suggesting that they may encode traits unique to the adherent and invasive phenotype (Nash *et al.*, 2010). Although these strains are closely related, each strain harbours a large plasmid that shows almost no conservation between them suggesting that they have different ancestry (Nash *et al.*, 2010). The plasmid in NRG857c is an antimicrobial resistance plasmid with a suite of genes encoding resistance to aminoglycosides, b-lactams, chloramphenicol, mercury, quaternary ammonium salts, sulfonamides, tetracycline, and trimethoprim (Nash *et al.*, 2010). LF82 and NRG857c genomes possess genes which encode for virulence associated factors such as type-VI secretion systems, iron acquisition, motility, adherence/invasion associated proteins such as type I pili, curli, biofilm formation, LPS production, transcriptional regulators of virulence genes, and alternative carbon source catabolism such as propanediol (a carbon source crucial to *Salmonella* virulence) (Miquel *et al.*, 2010; Nash *et al.*, 2010). Similarly, NRG857c contains genes that are important for adhesion and invasion of AIEC LF82, including *nlpI, htrA* and *dsbA* (Nash *et al.*, 2010). Interestingly, LF82 and NRG857c produce SPATE proteins (53% of *E. coli* isolated form CD patient) and adhesins (61.5% of *E. coli* isolated from CD patients) that are associated with UPEC (Kotlowski *et al.*, 2007). SPATE are serine protease autotransporter proteins that are key virulence genes in UPEC which have been grouped with AIEC strains through multi-locus sequence typing (MLST) analysis (Sepehri *et al.*, 2009). However, phylogenetic analyses of AIEC HM605 show that it is more divergent than NRG8578c and LF82 (which are of the same serotype O83:H1) and genomic comparisons suggest that there are few AIEC-specific regions and that the regions identified as characteristic of AIEC in a comparison of LF82 and NRG857c only reflect the close relationship of the two AIEC O83:H1 strains (Clarke *et al.*, 2011). An interesting feature of AIEC UM146 is that it possesses a range of resistance determinants that may help to explain the varied effectiveness in treating inflammatory bowel disease patients with antimicrobials (Krause *et al.*, 2010).

Table 1.3 General features of AIEC genomes

Strain	Phylogroup *	Chromosome Size (kb)	Chromosome G+C (%)	Chromosome Accession number	Plasmid Size (kb)	
LF82	B2	4,773	50.7	CU651637	108	
NRG8578c	B2	4,748	50.7	CP001855	147	
HM605	B2	4,989	51.8	CADZ01000001 to CADZ01000154	1. 135 (plasmid pS88)	2. 92 (plasmid pR3521)

* CD AIEC mainly associated with B2/D phylogroups (Martinez-Medina *et al.*, 2009c)

1.5 Pathoadaptive mutations

It has been well established that pathogens can evolve from non-pathogenic ancestors via gene acquisition by horizontal gene transfer, allowing these bacteria to colonise new niches. Furthermore, the bacterial cells core genes and newly acquired genes may undergo further genetic modifications to further enhance fitness levels in this new environment. This process is referred to as pathoadaptation (Sokurenko *et al.*, 1998). Pathoadaptive mutations occur in a many pathogenic bacteria. In *Shigella* spp. and EIEC a typical pathoadaptation is the inactivation of the *cad* locus which comprises the genes necessary for lysine decarboxylation, an enzyme involved in pH homoeostasis. This mutation allows these bacteria to survive and multiply within epithelial cells (Casalino *et al.*, 2009). Similarly, in UPEC the FimH adhesin has undergone genetic modification, enabling it to better bind to its ligand and therefore increase the ability of the bacteria to colonise the bladder (Sokurenko *et al.*, 1998). Interestingly, analysis of the LF82 genome sequence revealed a novel amino acid substitution in the *fimH* gene (T158P), which has not been identified in any *E.coli* strain sequenced to date (Miquel *et al.*, 2010). The position of this substitution is located in the flexible loop which connects the pilin and the lectin domains. Previous studies show that amino acid substitutions in this interdomain region increase the affinity (up to 300-fold) of FimH to its mannose receptor (Aprikian *et al.*, 2007).

Therefore it has been speculated that this substitution may enhance LF82 interaction with host cells. The analysis of the OmpA amino acid sequences in LF82 showed an OmpA variant having V110F and Y111D substitutions located at the top of the inflexible external L3 loop. This substitution is hypothesised to be involved in the recognition of a host cell receptor (Miquel *et al.*, 2010). Similarly, the OmpC and YfgL sequences in LF82 showed strong homology to other pathogenic *E. coli* such as CFT073 and ED1a compared to non-pathogenic *E coli* (Miquel *et al.*, 2010). The analyses of the LF82 genome clearly show that pathoadaptive mutations could play a major role in making AIEC pathogenic in a compromised host (Miquel *et al.*, 2010). Due to the varying genetic relationships between AIEC strains it has been proposed that AIEC are similar to ExPEC and have acquired genes and pathoadaptive mutations over time that allows them to take advantage of the micro-environment of a CD intestine (Sepehri *et al.*, 2009).

1.6 Objectives of this study

There were three main objectives of this study. The initial objective was to examine the relationship between AIEC and other commensal and pathogenic strains of *E. coli*. In accordance with this objective the aim was to establish the distribution of the AIEC phenotype (ability to adhere and invade epithelial cells and the ability to replication in macrophage) in the *E. coli* genus. Furthermore, as the phenotypic characteristics of AIEC are based on gene knockout experiments on the ileal AIEC strain LF82, a second objective of this study was to examine the role of two component pathways in the pathogenesis of the AIEC strain HM605. To achieve this attention was initially focused on the potential role of two component signal transduction pathways in intramacrophage replication and ability to adhere and invade epithelial cells. Finally, as little is still known how AIEC survive phagocytosis and intraphagolysosomal killing by macrophage, the final objective was to investigate the role of the oxidative stress response in the ability of AIEC HM605 to resist killing by macrophages.

Chapter 2.0 Materials and Methods

2.1 Bacterial strains and Plasmids

The bacterial strain *Escherichia coli* HM605 isolated from a colonic mucosal biopsy of a CD patient is referred to as the wild-type in this study (Martin *et al.*, 2004). The strains used in this study are presented in Table 2.1 and the plasmids in Table 2.2.

2.2 Cell lines and cell culture

2.2.1 J774.A1 murine macrophages

J774 is a mouse macrophage cell line that was derived from a tumour in a female BALB/c mouse and has been shown to possess characteristics of macrophages. The murine macrophage-like cell line J774 (American Type Culture Collection ATCC No. TIB67) was maintained in an atmosphere containing 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM), High glucose (25mM), GlutaMAX™ (L-Anayl-L-Glutamine, 3.97mM) (GIBCO) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich®), 100 U/ml penicillin and 100 μg/mL streptomycin (Sigma-Aldrich®).

2.2.2 C2Bbe1 epithelial cells

The C2Bbe1 cell line, which was sub-cloned from human colon adenocarcinoma Caco-2 cells, demonstrate features of enterocytic differentiation and form polarized monolayers with an apical brush border morphology comparable with that of human intestine were purchased from ATCC (CRL-2012) and maintained in an atmosphere containing 5% CO_2 at 37°C in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich®), 1% non-essential amino acids (Sigma-Aldrich®), 0.01mg/ml human transferrin (Calbiochem®) and 100 U/ml penicillin and 100 μg/mL streptomycin (Sigma-Aldrich®).

2.2.3 Acanthamoeba polyphaga

Acanthamoeba polyphaga was obtained from Dr Susan Joyce (Alimentary Pharmabiotic Centre (APC), Ireland) and grown in proteose peptone-yeast-glucose (PYG) medium as monolayers in 75 cm2 tissue culture flasks at 24°C. Amoebae were sub-cultured weekly by gently tapping flasks to detach cells before diluting 1: 10 in fresh medium. Stationary phase (3–5 day) cultures were used throughout this study.

2.3 Growth conditions

Luria-Bertani (LB) broth, MILLER (Merck) (5g yeast extract, 10g peptone from casein and 10g sodium chloride per litre of water) was used to culture all bacterial strains used in this study. Solid media was prepared by adding 1.5% (w/v) agar (Merck). Unless otherwise stated, *E. coli* cultures were incubated at 37^{o}C, either statically or shaking at 200rpm. Antibiotics were added to the media when required at the following concentrations; ampicillin (Amp) 100 µg/ml, chloramphenicol (Cm) 20 µg/ml, kanamycin (Km) 30 µg/ml and gentamicin (Gm) 20 µg/ml.

2.4 Overnight Starter Cultures

A single colony of the required bacterial strain was inoculated into a round bottom 20ml Sterilin tube containing 5ml LB using a sterile inoculating loop. Appropriate antibiotics were added as required. The culture was incubated shaking in a Grant OLS200 water bath at 37^{o}C or statically in an incubator at 37^{o}C. Glycerol stocks were made of each strain (750µl 40% (v/v) glycerol: 750µl overnight culture) in cryovials and were stored at -80^{o}C. At the end of each week strains were freshly restreaked from glycerol stocks onto solid media in preparation for the following week.

Table 2.1: List of strains relevant to this study

Strain	Relevant features	Source	Comments
ECOR 72 Library	Standard collection of 72 wildtype *E. coli*	Ochman, H. and Selander, R.K. 1984	Used in macrophage infection
E. coli MC4100	F^{-} [*araD139*], Δ(*argF-lac*)169, λ^{-} e14^{-} *flhD5301* Δ(*fruK-yeiR*), 725(*fruA25*), *relA1* *rpsL150*(Str^{R}), *rbsR22* Δ(*fimB-fimE*)632(::IS1) *deoC1*	Lab stock	*E. coli* K-12 control
E. coli W3110	λ, IN^{a} (*mD-rmE*)1, *rph1*	Lab stock	*E. coli* K-12 control
E. coli ZK2686	W3110 Δ(argF-lac)U169	Lab stock	*E. coli* K-12 control
E. coli CSH50	F^{-} λ^{-} *ara* Δ(lac-pro), *rpsL thi* *fimE::IS1*	Lab stock	*E. coli* K-12 control
E. coli A0 34/86	Wildtype porcine isolate (O83:K24:H31)	Lab stock	*E. coli* K-12 control
E. coli MG1655	F^{-} λ^{-} *ilvG- rfb*-50 *rph*-1	Lab stock	*E. coli* K-12 control
ΔcpxR::km	*ΔcpxR::km* knockout mutant in *E. coli* MG1655.	This study	Response regulator mutant in HM605

E. coli BW25113	Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), lambda$^-$, *rph-1*, Δ*(rhaD-rhaB)568, hsdR514*	Lab stock	*E. coli* K-12 control
ΔcpxR::km	*ΔcpxR::km* knockout mutant in *E. coli* BW25113.	This study	Response regulator mutant in HM605
E. coli HM229	*E. coli* isolated from a colonic mucosal biopsy of a colon cancer patient	Barry Campbell (Martin *et al.*, 2004)	Used in macrophage infection
E. coli HM454	*E. coli* isolated from a colonic mucosal biopsy of a healthy patient	Barry Campbell (Martin *et al.*, 2004)	Used in macrophage infection
E. coli HM358	*E. coli* isolated from a colonic mucosal biopsy of a cancer patient	Barry Campbell (Martin *et al.*, 2004)	Used in macrophage infection
E. coli HM605 (WT)	*E. coli* isolated from a colonic mucosal biopsy of a CD patient	Barry Campbell (Martin *et al.*, 2004)	The wild-type (WT) strain in this study
E. coli LF82	*E. coli* isolated from a patient with ileal CD	Arlette Darfeuille-Michaud (Boudeau *et al.*, 1999)	AIEC strain
WT + pCA24N-nlpE	*E. coli* HM605 complemented with pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Overproduction of *nlpE* to activate Cpx A/R pathway
WT + pUA66-*rpoE*-gfp	WT HM605 with *rpoE*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
WT + pUA66-*sinH*-gfp	WT HM605 with *sinH*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
WT + (pUA66-sinH::Km) + (pCA24N-nlpE::Cm)	WT HM605 with *sinH*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Used in gene expression analyses
WT + (pUA66-sinI::km)	WT HM605 with *sinI*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
WT + (pUA66-sinI::Km) + (pCA24N-nlpE::Cm)	WT HM605 with *sinI*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Used in gene expression analyses
WT + (pUA66-ycfS::Km)	WT HM605 with *ycfS*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses

WT + (pUA66-*ycfS*::Km) + (pCA24N-nlpE::Cm)	WT HM605 with *ycfS*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Used in gene expression analyses
WT + (pUA66-yebE::Km)	WT HM605 with *yebE*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
*Δcpx*R::km	*ΔcpxR*::km knockout mutant in *E. coli* HM605.	This study	Response regulator mutant in HM605
Δ cpxR	*ΔcpxR* markerless mutant in *E. coli* HM605	This study	Km^R removed by Flp recombinase
*Δcpx*R::km (pCA24N-cpxR::Cm)	*ΔcpxR*::km knockout mutant in *E. coli* HM605 complemented with pCA24N expressing a copy of *cpxR* (Cm^R)	This study	Complementation of *ΔcpxR::km* in HM605
*Δcpx*R::km (pCA24N-nlpE::Cm)	*ΔcpxR*::km knockout mutant in *E. coli* HM605 complemented with pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Overproduction of *nlpE* to activate Cpx A/R pathway
*Δcpx*R::km (pCA24N-*dsbA*::Cm)	*ΔcpxR*::km knockout mutant in *E. coli* HM605 complemented with pCA24N expressing a copy of *dsbA* (Cm^R)	This study	Overproduction of *dsbA* in *ΔcpxR::km* mutant of HM605
*Δcpx*R::km (pCA24N-*degP*::Cm)	*ΔcpxR*::km knockout mutant in *E. coli* HM605 complemented with pCA24N expressing a copy of *degP* (Cm^R)	This study	Overproduction of *degP* in *ΔcpxR::km* mutant of HM605
ΔcpxR (pUA66-rpoE::Km) + (pCA24N-dsbA::Cm)	*ΔcpxR* markerless mutant in *E. coli* HM605with *rpoE*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *dsbA* (Cm^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-rpoE::Km) + (pCA24N-degP::Cm)	*ΔcpxR* markerless mutant in *E. coli* HM605with *rpoE*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *dsbA* (Cm^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-rpoE::Km)	*ΔcpxR* markerless mutant in *E. coli* HM605with *rpoE*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-ycfS::Km) + (pCA24N-nlpE::Cm)	*ΔcpxR* markerless mutant in *E. coli* HM605with *ycfS*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE*	This study	Used in gene expression analyses

	(Cm^R)		
ΔcpxR::km (pBAD33-sinH::Cm)	*ΔcpxR*::km knockout mutant in *E. coli* HM605 complemented with pBAD33 expressing a copy of *sinH* (Cm^R)	This study	Complementation of *sinH* in *ΔcpxR::km* mutant of HM605
ΔcpxR (pUA66-sinH::km)	*ΔcpxR* markerless mutant in *E. coli* HM605with *sinH*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-sinH::Km) + (pCA24N-nlpE::Cm)	*ΔcpxR* markerless mutant in *E. coli* HM605with *sinH*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-sinI::Km)	*ΔcpxR* markerless mutant in *E. coli* HM605with *sinI*-gfp transcriptional promoter fusion (pUA66-Km^R)	This study	Used in gene expression analyses
ΔcpxR (pUA66-sinI::Km) + (pCA24N-nlpE::Cm)	*ΔcpxR* markerless mutant in *E. coli* HM605with *sinI*-gfp transcriptional promoter fusion (pUA66-Km^R) + pCA24N expressing a copy of *nlpE* (Cm^R)	This study	Used in gene expression analyses
ΔrcsB::Cm	*ΔrcsB*::Cm knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔzraR::km	*Δ zraR::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔypdB::km	*Δ ypdB::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔyehT::km	*Δ yehT::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔphoB::km	*Δ phoB::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔnarL::km	*Δ narL::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔuhpA::km	*Δ uhpA::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔompR::km	*Δ ompR::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔuvrY::km	*Δ uvrY::km* knockout mutant in *E. coli* HM605.	This study	Response regulator mutant
ΔrssB::km	*Δ rssB::km* knockout mutant in	This study	Response

	E. coli HM605.		regulator mutant
flgG::km	*E. coli* HM605 with Tn5 insertion within *flgG*::km	This study	Tn5 motility mutant of HM605
flgD::km	*E. coli* HM605 with Tn5 insertion within *flgD*::km	This study	Tn5 motility mutant of HM605
fliH::km	*E. coli* HM605 with Tn5 insertion within *fliH*::km	This study	Tn5 motility mutant of HM605
hemF::km	*E. coli* HM605 with Tn5 insertion within *hemF*::km	This study	Tn5 hydrogen peroxide sensitive mutant of HM605
katG::km	*E. coli* HM605 with Tn5 insertion within *katG*::km	This study	Tn5 hydrogen peroxide sensitive mutant of HM605
uvrD::km	*E. coli* HM605 with Tn5 insertion within *uvrD*::km	This study	Tn5 hydrogen peroxide sensitive mutant of HM605
uvrD::km (pCA24N-uvrD::Cm)	*E. coli* HM605 with Tn5 insertion within *uvrD*::km complemented with pCA24N expressing a copy of *uvrD* (Cm^R)	This study	Complementation of *uvrD::km* in HM605
ΔuvrB::Km	*ΔuvrB::km* knockout mutant in *E. coli* HM605.	This study	DNA repair mutant of HM605
Δnfo::Km	*Δnfo::km* knockout mutant in *E. coli* HM605.	This study	DNA repair mutant of HM605
ΔmutS::Km	*ΔmutS::km* knockout mutant in *E. coli* HM605.	This study	DNA repair mutant of HM605

Table 2.2: List of plasmids relevant to this study

Plasmids	Relevant features	Source	Comments
pFLP3	Source of Flp recombinase	Jerry Reen (Dept. Microbiology, University College Cork (UCC)	Used to remove kanamycin cassette from HM605Δ*cpxR*::km
pCA24N	Cm^R; *lacI* ^{q}, pCA24N	Baba *et al*., 2006	Used in plasmid based complementation
pCA24N-*cpxR*	Cm^R; *lacI* ^{q}, pCA24N $P_{T5\text{-}lac}$::*cpxR* $^+$	Baba *et al*., 2006	Used in plasmid based complementation
pCA24N-*nlpE*	Cm^R; *lacI* ^{q}, pCA24N $P_{T5\text{-}lac}$::*nlpE* $^+$	Baba *et al*., 2006	Used in plasmid based complementation

pCA24N-*dsbA*	Cm^R; *lacI* ^{q}, pCA24N $P_{T5\text{-}lac}$::*dsbA* $^+$	Baba *et al*., 2006	Used in plasmid based complementation
pCA24N-*degP*	Cm^R; *lacI* ^{q}, pCA24N $P_{T5\text{-}lac}$::*degP* $^+$	Baba *et al*., 2006	Used in plasmid based complementation
pCA24N-*uvrD*	Cm^R; *lacI* ^{q}, pCA24N $P_{T5\text{-}lac}$::*uvrD* $^+$	Baba *et al*., 2006	Used in plasmid based complementation
pUA66	Vector, SC101 ori, Km^R GFP reporter plasmid carrying *gfp*mut2	Zaslaver A., *et al* 2006	GFP expression vector
pUA66-*rpoE*	Vector, SC101 ori, Km^R GFP reporter plasmid carrying *gfp*mut2::*rpoE*	Zaslaver A., *et al* 2006	GFP expression vector
pUA66-*ycfS*	Vector, SC101 ori, Km^R GFP reporter plasmid carrying *gfp*mut2::*ycfS*	Zaslaver A., *et al* 2006	GFP expression vector
pUA66-*yebE*	Vector, SC101 ori, Km^R GFP reporter plasmid carrying *gfp*mut2::*yebE*	Zaslaver A., *et al* 2006	GFP expression vector
pUA66-*sinH*	Vector, SC101 ori, Km^R GFP reporter plasmid carrying *gfp*mut2::*sinH*	This study	GFP expression vector
pBAD33	Arabinose inducible expression vector, Cm^{R+}	Guzman et al. 1995	Used in plasmid based complementation
pBAD33-*sinH*	Arabinose inducible expression vector, Cm^{R+}::*sinH*	This study	Used in plasmid based complementation

2.5 Kits and Enzymes

Plasmids were purified using the QIAprep® Spin Miniprep kit (Qiagen) after overnight cultures were centrifuged for 15 min at 2,700rcf at 20^{o}C in a benchtop centrifuge. PCR and restriction products were cleaned using the Qiagen QIAquick® PCR purification kit (Qiagen) according to the protocol provided. Bands excised from 1% agarose gels were placed in a 1.5ml microcentrifuge tube, weighed and then cleaned using the QIAquick® Gel Extraction Kit (Qiagen) according to the protocol provided. For the extraction of RNA, cultures were first treated with RNAprotect™ (Qiagen) before RNA was extracted using the RNAeasy ® Mini kit

(Qiagen) (see section 2.11). Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich®) (see section 2.6). Enzymes were supplied by New England Biolabs (NEB) or Promega.

2.6 Electrophoresis

Gels containing 1.0% agarose in 1 x TAE (4.84g Tris, 1.14ml glacial acetic acid, 2ml 0.5M EDTA pH 8.0 per litre of sdH_20) and 5µg/ml Ethidium Bromide (Sigma®) were prepared for the electrophoresis of DNA, with running buffer 1 x TAE. Gels were compared against Promega 1kb ladder as a reference and run at 100mV for 30 minutes. For RNA, gels containing 1.5% agarose in 1 x TBE (Ambion) and 5µg/ml Ethidium Bromide (Sigma®) were prepared. Gel units were cleaned with RNase wipes (Ambion) and rinsed in dH_20 before use. RNA gels were loaded with Low Molecular Weight DNA ladder (NEB) as a reference and run in a 1 x TBE running buffer for 30 min at 200mV.

2.7 Genomic DNA Extraction

Genomic DNA from the bacterial strain of interest was extracted using methods described in the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich®). All centrifugation steps were carried out at room temperature unless otherwise stated. An overnight culture of the bacterial strain of interest was grown to saturation. A 1.5ml sample from the overnight culture was transferred to a 2ml microcentrifuge tube before being spun in a centrifuge for 2 min at 11,000rcf. The supernatant was discarded and the pellet resuspended in 180µl Lysis Solution T before adding 20µl of 20mg/ml Proteinase K, mixed and incubated at 55 ^oC for 30 min. After which time 200µl of Lysis Solution C was added, mixed and incubated at 55 ^oC for 10 min. At this stage, the binding column was prepared by adding 500µl of Column Preparation Solution to each column before being spun in a centrifuge for 1 min at 9,000rcf. The flow-through was discarded. After lysis of cells, 200µl of ethanol was added, mixed and the contents of the tube were added to the pre-treated binding column and spun for 1 min at 7,000rcf. The flow through was discarded and the column was transferred to a new collection tube where 500µl of Wash Solution 1 was added to the column and spun at 7,000rcf for 1 min. The flow through was discarded and the column was again placed in a new collection tube and 500µl of Wash Solution Concentrate was added to the column and spun at 9,000rcf for 3

minutes to dry the column. Finally, the column was transferred to a 2ml collection tube, 200µl of sterile water was added to the column's membrane before spinning at 7,000rcf for 1 min to elute DNA that was then stored at -20^{o}C. A 5µl sample of DNA was then run on a 1% agarose gel.

2.8 Primer List

All primers were ordered from MWG Biotech (Germany) and resuspended in sterile H_2O to a concentration of 100pmol/µl.

Table 2.3: List of primers used in this study

Primer	Sequence (5'-3') (including restriction sites highlighted in bold)	T_m ^{o}C	Description
ARB1	GGCCACGCGTCGACTAGTTACNNNNNNNNNNGATAT	73.2	Arbitrary PCR
ARB2	GGCCACGCGTCGACTTAGTTAC	60.1	Arbitrary PCR
ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	76.7	Arbitrary PCR
Tn5int	GAATATGGCTCATAACACCC	50.6	Arbitrary PCR
Tn5ext	CCTGATTGCCCGACATTATCGCG	60.4	Arbitrary PCR
KtpKD4	CGGCCACAGTCGATGAATCC	58.4	Km^R cassette
K2pKD4	CGGTGCCCTGAATGAACTGC	59.2	Km^R cassette
pBAD33-sinH Fw	TTAT**GAGCTC**CATTGTCACAGGGAAGTGCATGCTGCGATGGAAACGC	69.9	Forward primer for cloning *sinH* into pBAD33
pBAD33-sinH Rv	TTAT**TCTAGA**GACTGGCCTTACGCCAATCTTATTTTTTGGTATACAGCACGC	65.9	Reverse primer for cloning *sinH* into pBAD33
cpxPF	TTAT**CTCGAG**GCGACAGAAAGATTTTGGAGC	68.2	Forward primer for *cpxP*
cpxPR	TTAT**GGATCC**CAGGGATGGTGTCTATGGCAAG	69.5	Reverse primer for *cpxP*
OL4	GACAGAACAACGATTGCCAG	54.2	PCR switch assay
OL20	CCGTAACGCAGACTCATCCTC	59.8	PCR switch assay

2.9 Electrocompetent *Escherichia coli*

One hundred ml of LB broth was inoculated with 200µl of an overnight culture and allowed to grow at 37°C to mid-exponential phase (OD_{600}=0.5-0.7). Cells were harvested by centrifugation at 2,700rcf for 10 min at 4°C. Supernatant was discarded and pellet was resuspended in 100ml ice-cold HPLC water. Cells were harvested as previously described and resuspended in 50ml ice-cold HPLC water. Again, cells were harvested and resuspended in 1.6ml ice-cold sterile 20% glycerol. Finally, cells were harvested and resuspended in 160µl ice-cold sterile 20% glycerol and frozen at –80°C. 50 µl cells were used per electroporation.

2.10 Construction of HM605 Tn5 mutant library

Electrocompetent cells were transformed with the EZ-Tn5 transposome® (Epicentre®), according to the manufacturers protocol, allowing for random insertion of the transposon. The electroporation mix was plated onto LB plates supplemented with 50µg/ml kanamycin and incubated at 37°C overnight. Colonies were picked into well plates containing 150µl LB freezing buffer (36mM K_2HPO_4, 13.2mM KH_2PO_4, 1.7mM sodium citrate, 0.4mM $MgSO_4$, 6.8mM $(NH_4)_2SO_4$, 44% glycerol) supplemented with 50µg/ml kanamycin and stored at –80°C.

2.11 Screening for motility minus mutants in *E. coli* HM605 Tn5 mutant library

To facilitate high-throughput analysis, a 5,568 Tn5 transposon mutant library of *E. coli* HM605 (see section 2.10), grown in wells of a 96-well polystyrene plate (Genetix®) was hand replicated onto Q-trays (Genetix®) containing 250ml of 0.3% swim motility agar (see table 2.4) using a 96-well pin replicator (Boekel®) . Plates were incubated overnight in the dark at 37°C. Motility was examined visually and colonies showing reduced motility were examined in secondary and tertiary screens. Transposon insertion sites were determined by Arbitrary primed PCR (see section 2.13).

2.12 Screening for hydrogen peroxide sensitive (H_2O_2) mutants in *E. coli* HM605 Tn5 mutant library

To facilitate high-throughput analysis, a 5,568 Tn5 transposon mutant library of *E. coli* HM605 (see section 2.10), grown in wells of a 96-well polystyrene plate (Genetix®). The mutants were transferred to a new 96 well plate containing LB

broth supplemented with 0.6mM H_2O_2 using a 96-well pin replicator (see Fig 2.1). Plates were incubated overnight at 37°C and OD_{600} readings were taken to determine growth. Mutants showing growth < 0.1 OD_{600} were considered H_2O_2 sensitive. Transposon insertion sites were determined by Arbitrary primed PCR (see section 2.13).

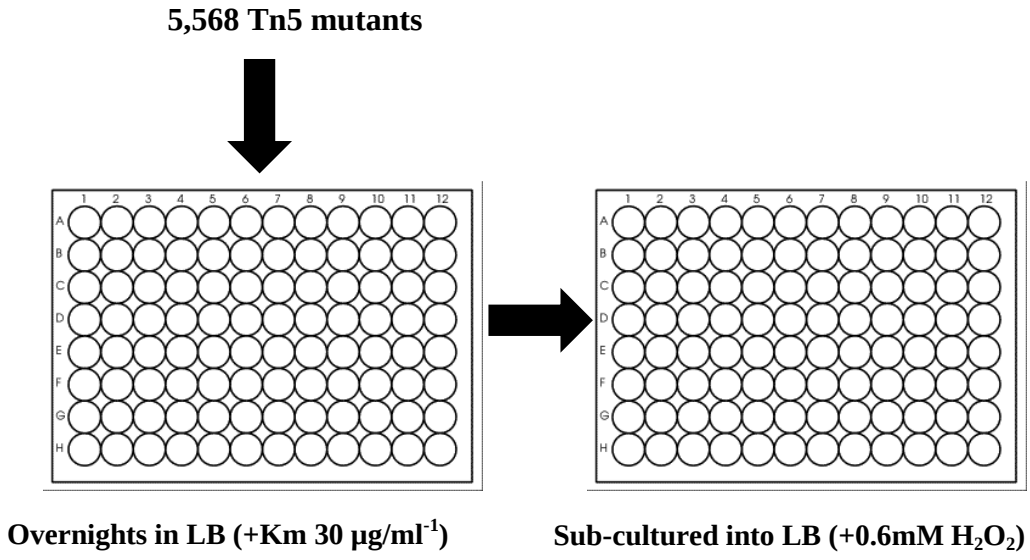


Figure 2.1: Work flow diagram for each HM605 Tn5 mutant. 5,568 mutants were grown overnight in LB supplemented with kanamycin (30 µg/ml^{-1}) in wells of a 96-well polystyrene plate. Using a 96-well pin replicator, the mutants were transferred to a new 96 well plate containing LB broth supplemented with 0.6mM H_2O_2 to identify H_2O_2 sensitive mutants.

2.13 Arbitrarily primed PCR

DNA was isolated from mutants using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich®) (see section 2.7). Transposon insertion sites were determined by amplifying the region within the genome surrounding the transposon using two rounds of arbitrarily primed PCR. Primers ARB1, ARB6 and Tn5ext were used in round one. Reaction mix is as follows: 1pmol/µl each primer, 10X buffer number 2 for KOD polymerase, 25mM $MgCl_2$, 10mM each dNTP, 0.5U KOD polymerase and

5µl genomic DNA. Cycling conditions were as follows: 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 56°C, 1 min at 72°C and 5 min at 72°C. PCR product was purified using the QIAquick PCR purification Kit (Qiagen). Primers ARB2 and Tn5int were used in round two. Reaction mix was identical to that used in round one, except 5µl of purified PCR product from round one was used as a template. Cycling conditions were also identical to round one, with an annealing temperature of 50°C for 30 sec. Products were purified as above and sequenced. To determine which gene was disrupted, sequences obtained from the PCR products were compared to sequences in the NCBI database using a BLAST search.

2.14 Minimum inhibitory concentration (MIC) determination of *E. coli* HM605 to hydrogen peroxide

A spectrophotometric assay for the minimum inhibitory concentration was determined in a sterile 96 well flat bottom polystyrene plate (Genetix®). Overnight cultures grown in LB broth were adjusted to an OD_{600} nm of 0.5 in 150µl of LB broth in a 96 well plate. Using a 96-well pin replicator, diluted bacteria were transferred to a new 96 well plate containing LB broth with various concentrations of hydrogen peroxide (H_2O_2) ranging from 0.3mM to 0.8mM (increasing in intervals of 0.05). Control wells contained LB broth with no H_2O_2 (positive control). Plates were incubated in the dark at 37°C for 24 h. To determine the minimum inhibitory concentration after 24 hours the optical density was determined at 600nm. The MIC is reported as the lowest concentration of test material which results in 100% inhibition of growth of the test organism.

2.15 Sensitivity of *E. coli* strains to hydrogen peroxide.

Sensitivity to H_2O_2 induced oxidative stress was determined by an agar overlay diffusion method on LB agar previously described with some modifications (Bertrand *et al.*, 2010). Overnight cultures grown in LB broth were adjusted to an OD_{600} nm of 0.5. 100µl of each culture was suspended in 4 ml of LB soft agar (0.5% agar) and poured over the LB agar plates. Sterile blank disks were added to the surfaces of the solidified overlays and 10µl of H_2O_2 (30%) was spotted onto the disks. The plates were then incubated overnight at 37°C and the diameters of inhibition zones were measured.

2.16 Catalase test

The catalase test facilitates the detection of the enzyme catalase in bacteria. A small amount of a well isolated 18- to 24-h colony was placed onto a microscope slide. 1-2 drops of 3% H_2O_2 was added and the formation of bubbles was assessed.

2.17 Bacterial stress tolerance

Bacterial cultures were grown at 37^{o}C in LB medium to an OD600nm= 0.1 and 5 µl samples were spotted onto LB agar plates containing 1 mM IPTG, 50 µg/ml kanamycin or 20 µg/ml Chloramphenicol when appropriate, and one of the following stress agents: 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.0, 100 mM MES pH 5.0 + 1 mM Sodium nitrite ($NaNO_2$), Plates were incubated overnight at 37^{o}C.

2.18 Identification of hydrogen peroxide-sensitive mutant strains of *E. coli* HM605

A 5,568 Tn5 transposon mutant library of *E. coli* HM605 (see section 2.10) was screened for mutants showing reduced growth in hydrogen peroxide. In brief, cultures of individual colonies were grown in 96-well microtitre plate (Genetix®), replica plated into fresh LB broth and LB broth containing 0.6mM hydrogen peroxide. To determine growth after 24 h the optical density was determined at 600nm. Wells that exhibited poor growth in the presence of hydrogen peroxide were presumptive hydrogen-sensitive mutants. These potential mutants were further examined for reduced growth in secondary and tertiary screens. Transposon insertion sites were determined by Arbitrary primed PCR (see section 2.13).

2.19 Determination of *fim* switch orientation

The method used to determine the *fim* switch orientation on the chromosome has previously been described (Smith & Dorman, 1999). The orientation in the chromosome of the 314 bp DNA segment harbouring the fimA promoter was determined using a PCR-based assay. This method exploited a unique *BstUI* restriction site in the *fim* switch that results in restriction fragment length dimorphism among *BstUI*-digested PCR products that are characteristic of phase-on and -off switches. Individual bacterial colonies were harvested from plates following overnight incubation at 37°C with bacteriological loops and resuspended in 100µl of

sterile distilled water. This suspension was boiled for 5 min to release genomic DNA. The switch region was amplified with oligonucleotides OL4 and OL20 (Table 2.3) to generate a 726 bp DNA product. DNA was amplified with Taq polymerase (Promega), using the following PCR cycle: denature at 94°C for 3 min followed by 30 cycles (94°C for 1 min, 58°C for 1 min and 72°C for 1 min) and a final extension of 10 min at 72°C. Samples were cooled to 60°C, 10 units of *BstUI* were added to each reaction and incubation was continued at 60°C for 3 h. Digested PCR products were electrophoresed on 2% agarose gels. Using this assay, phase ON populations of bacteria yielded two DNA fragments 433 and 293 bp in length; whereas phase OFF populations yielded two fragments of 539 and 187 bp (see Figure 2.2). Mixed populations contained a mixture of all four fragments.

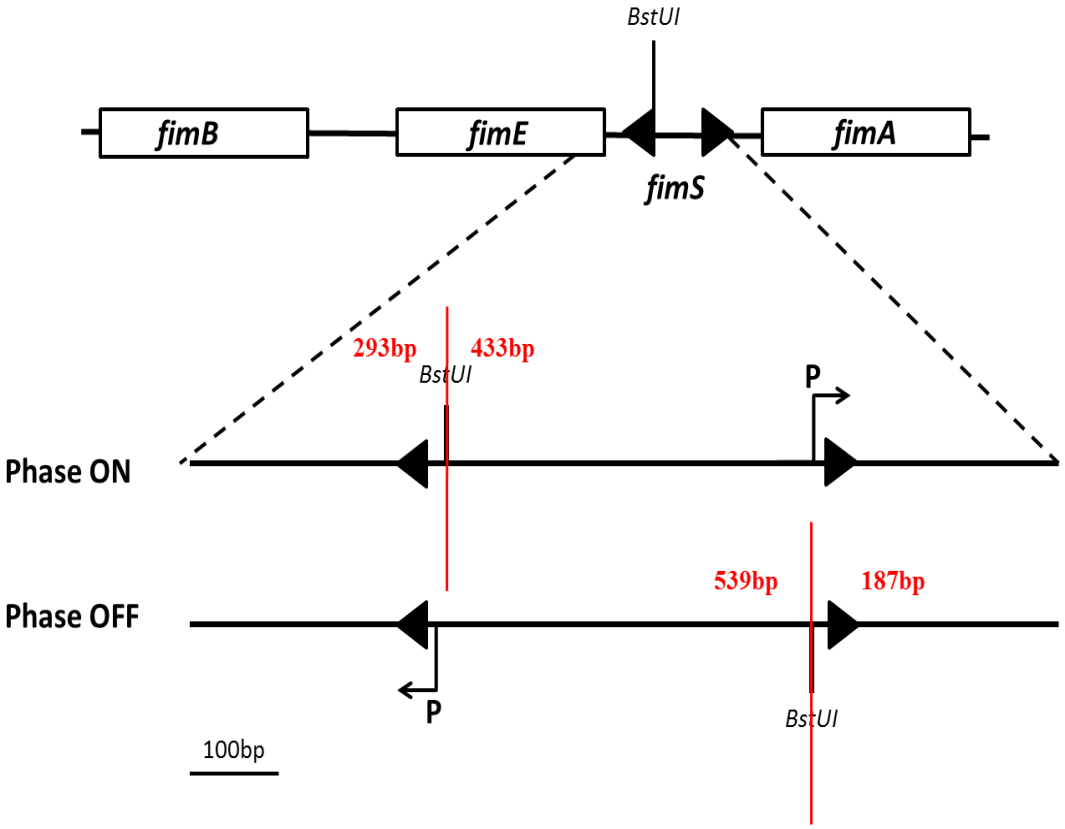


Fig. 2.2: Diagrammatic representation of the *fim* switch orientation showing the phase -ON and –OFF switches, the location of the *BstUI* mobile restriction site and the *fimA* promoter (modified from Smith and Dorman 1999).

2.20 Yeast aggregation assay

To test the production of type 1 fimbriae, mannose-sensitive yeast agglutination assays were performed as described previously (Bringer, 2007). Commercial baker's yeast (*Saccharomyces cerevisiae*) was suspended in PBS to a final concentration of 4mg (dry weight) /ml. Bacterial strains were grown overnight statically in LB broth at 37°C, washed and resuspended in PBS to an OD_{600} nm of 0.5. Equal amounts of yeast suspension and bacterial suspension were mixed in a 24-well polystyrene plate (Sartstedt®) and aggregation was monitored visually.

2.21 Flp recombinase-mediated marker excision from chromosomally-inserted DNA

Site-specific recombinases, usually yeast Flp recombinase or bacteriophage P1 Cre recombinase, can be used for site-specific excision (deletion) of DNA fragments flanked by the cognate recombination sites, i.e. Flp recombinase target (*FRT*) sites for Flp (see Fig 2.3). Excision of antibiotic selection markers allows recycling of the same resistance determinants in subsequent genetic manipulation procedures. The plasmid pFLP3 (see table 2.1) is a tetracycline and ampicillin resistant plasmid showing induction of FLP recombinase. To mediated marker excision, pFLP3 was transformed into a kanamycin resistant *E. coli* HM605 strain containing a chromosomally integrated kanamycin cassette and incubated at 37°C until single colonies were visible. Colonies were then screened for tetracycline resistance indicating uptake of plasmid. The plasmid pFLP3 is curable from its host by $sucrose^R$ selection since it carries the counter-selectable *sacB* marker. pFLP3 was cured by generously streaking cells onto LB containing 5% sucrose followed by incubation at 37°C. Loss of pFLP3 was tested by patching $sucrose^R$ colonies on a tetracycline resistant agar plate, as well as a kanamycin resistant agar plate to confirm excision of kanamycin cassette and a plain LB agar plate. Growth on only the plain LB agar plate indicates loss of plasmid and excision of kanamycin cassette. Excision events were initially assessed by performing colony PCR on kanamycin sensitive colonies using two primers, k2pKD4 and ktpKD4 (see table 2.3) to confirm loss of kanamycin marker.

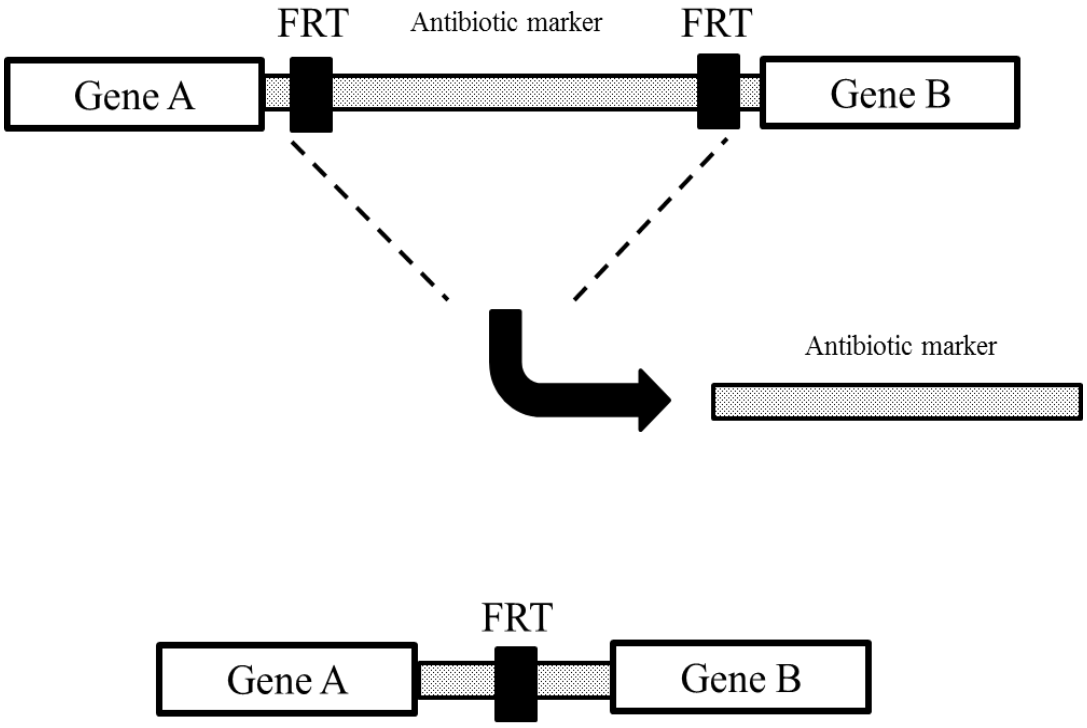


Figure 2.3: Diagrammatic representation of Flp recombinase-mediated marker excision from chromosomally-inserted DNA. The antibiotic resistance marker is flanked by Flp recombinase target FRT sites which allow the antibiotic marker to be eliminated with the use of a FLP expression plasmid.

2.22 Construction of deletion mutants in *E. coli* using P1 transduction

This method of creating deletion mutants in *Escherichia coli* has been previously described (Silhavy *et al.*, 1984). This technique relies on the transfer of genetic material from a donor strain to a recipient strain via the bacteriophage P1*cml* or P1*kml*. Donor strains containing the desired mutant allele were obtained via the keio collection (Baba *et al.*, 2006). The keio collection comprises a set of single-gene in-frame deletion mutants of most non-essential genes (3,985) of E. coli and therefore provides a useful resource for the transfer of mutant alleles to recipient strains via P1 transduction. The Keio collection was constructed on *E. coli* K-12 BW25113 (*lacI^{q}rrnBT14, ΔlacZWJ16, hsdR514, ΔaraBADAH33, and ΔrhaBADLD78*) (Baba *et al.*, 2006)

2.22.1: Preparation of Lysogens

Three colonies of the donor strain (from keio collection) were re-suspended in 50µl of LB Broth. 50 µl of previous phage lysate (from wild-type *E. coli* strain) and 5 µl $CaCl_2$ (1 M) was then added. The sample was incubated in a 30^{o}C water-bath for 30 min. The whole suspension was plated on LB agar supplemented with Chloramphenicol or Kanamycin and incubated overnight at 30^{o}C. One colony of the lysogen was purified by re-streaking it on Chloramphenicol or Kanamycin LB plates.

2.22.2: Preparation of high titre P1 c/kml lysate

2 ml of LB (+Cm or +Km) was inoculated with a single colony and incubated at 30^0 C overnight. The overnight culture was then used to inoculate 5 ml of LB Broth supplemented with 5 µl of Chloramphenicol or Kanamycin to an OD_{600}nm = 0.02. A 1: 100 dilution of the culture was carried out therefore 50 µl of the culture was added to 5 ml of LB Broth. The sample was then incubated and let grow at 30^{o}C until the OD_{600}nm=0.1 to 0.2 (~2h). The sample was heat-shocked by incubation at 42^{o}C for 20 min without agitation (vigorously shaken for 3-4sec during incubation). The P1 phage is in the chromosome of the bacteria and the heat-shock step brings the phage out of the chromosome and allows it to begin replicating and begin to lyse the cells. The culture was incubated at 37^{o}C with agitation until the cells had lysed (~2 h). 100 µl of Chloroform was added to the culture and the whole suspension centrifuged at

2,700rcf for 10 min at 4^{o}C. The supernatant was transferred to a 15ml tube and 100 µl of Chloroform was added. The Lysate was stored at 4^{o}C.

2.22.3 Transduction

5 ml of LB Broth was inoculated with the recipient and incubated overnight at 37^{o}C. The cells were centrifuged at 2,700rcf for 10 min and re-suspended in 2.5 ml $MgSO_4$ (10mM), $CaCl_2$ (5mM). A number of volumes of phage lysate (10µl, 50µl and 100µl) were added to 100 µl of recipient cells. A control used consisted of recipient strain and LB only (no phage lysate). Samples were incubated at 37^{o}C for 30 min WITHOUT AGITATION. 200µl of Sodium Citrate (1 M) and 1 ml of LB Broth (pre-warmed at 37^{o}C) was added to each sample and incubated at 37^{o}C for 1 h. Centrifugation at 11,000rcf for 1 min was used to pellet the cells and 100µl of LB containing 20mM Sodium Citrate was used to re-suspend the cells. 50µl Sodium Citrate (1 M) was spread onto selective medium to induce transduction. The cells were plated onto the selective medium and incubated overnight at 37^{o}C. One colony was purified from each on selective plates.

2.23 Colony PCR for kanamycin cassette

Following P1 transduction, a colony PCR was used to confirm the presence of the kanamycin cassette. Primers ktpKD4 and k2pKD4 were used. Reaction mix is as follows: 2pmol/µl each primer, 5X Green GoTaq buffer, 10mM each dNTP, 0.5U Taq polymerase. Template for the reaction consisted of 1µl of one colony suspended in 50µl sterile HPLC water. Cycling conditions were as follows: 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 56°C, 1 min at 72°C and 5 min at 72°C.

2.24 Plasmid based complementation using pCA24N

An attempt was made to complement all knock out mutants using a plasmid based complementation method. The complementation vector routinely used in the complementation of *E. coli* deletion mutants is pCA24N (see Table 2.1) from the ASKA library. The ASKA library is a complete set of open reading frame clones of *E. coli* K-12. The ASKA library contains 85% of *E. coli* K-12 ORFs. The ORFs were amplified by PCR and cloned between *SfiI* restriction sites. The ASKA plasmids use the high copy number plasmid vector pCA24N (Kitagawa *et al.*, 2005).

Expression of cloned genes is directed by the $P_{T5\text{-}lac}$ promoter that can be activated by IPTG but normally repressed by a copy of *lacI*^{q} on the plasmid. The ASKA set has been used for a wide variety of genomics applications, including complementation and overexpression phenotypes (Arifuzzaman *et al.*, 2006; Sugihara *et al.*). To validate the results obtained by knockout mutants, plasmids encoding the knocked-out genes were extracted from colonies in the ASKA library and transformed to their respective strains (see Section 2.9).

2.25 Plasmid based complementation using pBAD33

For complementation into pBAD33, cloned genes are inserted under the control of an arabinose pBAD-inducible promoter. Primers were designed to amplify the target region for cloning but to also include a tail which was engineered to contain *SacI* and *XbaI* restriction sites. Gene *sinH* was amplified from HM605 using 0.25 units Phusion polymerase, 10mM dNTP's, 5X HiFi Buffer, 1pmol/µl pBAD33-*sinH* Fw, 1pmol/µl pBAD33-*sinH* Rv in a total volume of 50µl. Cycling conditions were as follows: 2 min at 98°C; 30 cycles of 30 sec at 98°C, 30 sec at 63°C, 15 sec at 72°C and 5 min at 72°C. The DNA fragment was digested with *SacI* and *XbaI* and was ligated to the *SacI* and *XbaI* sites in the previously digested pBAD33 vector.

2.26 Phenotypic characterisation

2.26.1 Swim motility agar

In order to assess swimming motility, 0.3% (w/v) LB agar was freshly prepared on the day of the assay. In order to reduce the amount of condensation on the lid this agar was allowed to cool significantly before being poured onto 90mm Petri dishes. In addition, plates were left to solidify with lids on in order to retain moisture. An overnight culture was diluted to an OD_{600}nm of 1.0 and 5µl inoculated onto the surface of the agar. For *E. coli* HM605 the agar had to be pierced in order for motility to be observed. Inoculated plates were left to dry before transferring to the 37^o C incubator, ensuring that plates were kept level at all times. After 24 hours incubation swim motility could be scored by the appearance of a swim halo surrounding the colony.

Table 2.4 Composition of broth and agars used in this study

*** Media was sterilised at 121 ^{o}C for 15 minutes and allowed to cool before use**

Media	Composition	Purpose
LB broth*	12.5g LB granules (Merck) 500ml dH_2O	Routine culturing
LB agar*	12.5g LB granules (Merck) 7.5g agar (Merck) 500ml dH_2O	Routine culturing
LB freezing buffer*	36mM K_2HPO_4 (anhydrous) 13.2 mM KH_2PO_4 1.7 mM sodium citrate 0.4 mM $MgSO_4$ 6.8 mM ammonium sulphate 4.4% (v/v) glycerol in LB broth	Storage of Tn5 transposon library
5% Sucrose agar*	5.0g Tryptone 2.5g Yeast extract (Bacto™) 25g Sucrose (Sigma-aldrich) 7.5g agar (Merck) 500ml dH_2O	Used to select for colonies that have lost plasmid pFLP3 in construction of a *E. coli* HM605*ΔcpxR* markerless mutant
Swim motility agar (0.03% (w/v) agar) *	12.5g LB granules (Merck) 1.5g agar (Merck) 500ml dH_2O	Used to determine swim motility and freshly prepared on day of use
PYG broth*	7.5g proteose peptone (oxoid L85) 9g D-glucose (Sigma) 1.25g Yeast extract (Merck) 500ml dH_2O	Routine culturing of *A. polyphaga*
PYG agar*	7.5g proteose peptone (oxoid L85) 9g D-glucose (Sigma) 1.25g Yeast extract (Merck) 7.5g agar (Merck) 500ml dH_2O	Used for *A. polyphaga* grazing resistance assays
Page's Amoeba Saline (PAS)	Combine 5ml of solution 1 and 5ml of solution 2 in 1L of sdH_2O*: **Solution 1**: 500ml: 12g NaCl,0.4g $MgSO_4$, 0.6g CaCl2.6H2O **Solution 2:** 500ml: 14.2g Na_2HPO_4, 13.6g KH_2PO_4	Used for coinfection assays of *A. polyphaga*

2.27 Growth

2.27.1 Measuring growth in conical flasks

Overnight culture was used to inoculate 50ml of LB broth (containing appropriate antibiotics) to OD_{600} nm 0.05 in a 200ml conical flask. Each strain was represented by 3 individual overnights and hence 3 individual cultures in the growth curve. Inoculated cultures were grown shaking in a 37^{o}C incubator at 200rpm. OD_{600} nm readings were taken every 30 min by diluting 1:10 or 1:20 in LB broth as necessary and reading absorbance using a spectrophotometer.

2.27.2 Measuring growth using MWG Sirius HT growth curve machine

Overnight cultures were diluted in fresh LB broth (containing appropriate antibiotics) to OD_{600} nm 0.05 and 150µl of diluted culture was inoculated into wells of a 96 well flat bottom translucent Sarstedt® plate or a 96 well black optical bottom Nunc® plate for *gfp* expressing strains. (50µl) of Mineral oil was added to each well to prevent condensation build up and evaporation of culture. The MWG Sirius HT machine was programmed to incubate the plates at 37^{o}C and to take an OD_{600} nm reading every 15 min over a period of 24 h.

2.27.3 Measuring growth at low pH using MWG Sirius HT growth curve machine

Overnight cultures were diluted in M9 minimal media (pH 4.5) supplemented with 0.02% (w/v) glucose and buffered with 100mM MES (containing appropriate antibiotics) to OD_{600} nm 0.05, 150µl of diluted culture was inoculated into wells of a 96 well flat bottom translucent Sarstedt® plate. 50µl of mineral oil was added to each well to prevent condensation build up and evaporation of culture. The MWG Sirius HT machine was programmed to incubate the plates at 37^{o}C and to take an OD_{600} nm reading every 15 min over a period of 12 h.

2.27.4 Measuring growth in the presence of Copper sulphate ($CuSO_4$) using MWG Sirius HT growth curve machine

Overnight cultures were diluted in fresh LB broth (containing appropriate antibiotics) to OD_{600} nm 0.05, 150µl of diluted culture was inoculated into wells a 96 well black optical bottom Nunc® plate. Cultures were incubated until OD_{600} 0.2 and

0.5mM $CuSO_4$ was added to the appropriate wells. The cultures were again incubated at 37^{o}C and OD_{600} and *gfp* fluorescence (excitation 485/20nm, emission 528/20nm) readings were taken every 15 min over a period of 24 h using a MWG Sirius HT plate reader.

2.28 Copper sensitivity test

Copper sensitivity test was performed as previously described (Yamamoto & Ishihama, 2006) to examine sensitivity of *ΔcpxR* mutants to external copper. Overnight cultures were diluted to OD_{600} =1.0 in fresh LB. The diluted culture was then serial diluted in 1 X PBS and plated onto LB agar containing 0.8 mM copper sulphate ($CuSO_4$) to determine the number of colony forming units (cfu).

2.29 Gentamicin protection assay

Macrophage survival assay was modified from a previously described method (Subramanian, 2008). J774.A1 cells were seeded into 24-well tissue culture plates at 4 x 10^5 per well and maintained in DMEM with 10% Fetal Bovine Serum and used 20 hours later. The monolayers were washed twice with sterile phosphate buffer saline (PBS). Prior to infection of cells, bacteria were washed three times, resuspended in antibiotic free DMEM and added to cells at a multiplicity of infection of 10 (4 x 10^6 cfu/ml). Following a 2 h incubation at 37^o C (5% CO_2) to allow internalization, cell monolayers were washed three times in PBS to remove nonadherent bacteria and treated with fresh culture medium containing 20µg/ml gentamicin for 1 h to kill extracellular bacteria. Following this the cells were washed three times with PBS and cells were lysed by adding 500µl ice cold water containing 1% Triton-X 100 for 5 min to release internalized bacteria. Tenfold dilutions of cell lysates were made and 100µl from each dilution was plated onto LB agar plates. Parallel plates were incubated at 37^0 C and colony forming units (CFU) were counted after 6h as a measure of viable bacteria. Survival/replication measured by comparing 6h post-gentamicin to 1h post-gentamicin.

2.30 Addition of vacuolar-pH-neutralizing reagent to infected cells

Ammonium chloride (NH_4Cl; Sigma-Aldrich®) at a concentration of 2mM was used to neutralize the vacuolar pH. Macrophages were infected as described above. The reagent was added to cells at 1 h post-infection. Infected and untreated cells were

analysed in parallel. All experiments were performed in triplicate. Results are expressed as the number of viable intracellular bacteria at 6 hours post-infection relative to that obtained at time 0, taken as 100%.

2.31 Detection of cytokines IL-1β and TNF-α in the supernatant of infected macrophages.

Macrophages were seeded and infected as described above. At time 1h and 24 h post gentamicin, supernatants were collected and stored at -20 ^{o}C. The amounts of murine cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) released in the culture supernatant or remaining in cells was analysed using Meso Scale Discovery technology (MSD® Meso Scale Discovery, Gaithersburg, MD) (MSD® 96 well MULTI-ARRAY® mouse cytokine assay) according to the manufacturer's instructions. In brief, 25µl of each calibrator and sample solution were dispensed into separate wells of the MSD® plate. Following this, the plate was sealed and incubated for 2 h with vigorous shaking (300rcf) at room temperature to ensure rapid binding of the sample to the capture antibodies. After this time, 25µl of the 1X Detection Antibody Solution was dispensed into each well. The detection antibodies are provided premixed in solution at a 50X concentration and the working detection antibody solution should be prepared at 1X (or 1.0 µg/ml). The plate was again sealed and incubated for 2 h with vigorous shaking (300rcf) at room temperature. Finally, each well was washed three times with PBS+0.05% Tween-20 followed by the addition of 150µl of 2X Read Buffer T to each well of the plate. The plate was then analysed on the SECTOR® Imager. Cytokine concentrations in picograms per millilitre (pg/ml) were assessed according to the manufacturer's instructions.

2.32 Epithelial cell adhesion and invasion assay

Epithelial cell adhesion and invasion assay was performed as previously described (Martinez-Medina *et al.*, 2009c). Monolayers were seeded in triplicate 24 well tissue culture plates with $4x10^5$ C2Bbe1 cells per well and incubated for 20h at 37°C /5% CO_2. Monolayers were then infected in 1ml of DMEM with 10% Fetal Bovine Serum without antibiotics at a multiplicity of infection of 10 bacteria per epithelial cell. After addition of bacteria cells were centrifuged for 5 min at 300rcf to allow closer interaction of bacteria and epithelial cells. After 3h infection, the monolayers of one plate were washed twice in sterile 1X PBS, lysed with 1% triton X-100,

serially diluted and plated onto LB plates to determine the number of colony forming units (cfu) corresponding to the total number of cell associated bacteria (adherent and intracellular bacteria). To determine the number of intracellular bacteria, cell culture medium containing 100µg/ml gentamicin was added to the second plate for 1hr to kill extracellular bacteria. Monolayers were then washed twice with sterile 1X PBS, lysed with 1ml of ice cold 1% triton X-100, and the bacteria were quantified as described above.

2.33 Amoeba grazing resistance

Amoeba grazing resistance assay was adapted from a previously described method (Adiba *et al.*, 2010). Amoebae were harvested by gently tapping flask, followed by centrifugation at 300rcf for 5min and washing twice in Page's Amoeba Saline Solution (PAS) before numbers were adjusted to 1 x 10^2 cells per ml using a haemocytometer. A volume of 300µl of a high bacterial population size (1 x 10^8 cells per ml) was plated on proteose peptone-yeast-glucose (PYG) agar and allowed to dry for 20 min in a laminar flow hood. The same volume of amoeba culture adjusted to 1 x 10^2 cells per ml was added on these plates and allowed to dry for 20 min in a laminar flow hood. Plates were covered with a parafilm and incubated for six days at 24°C. Three replicates were performed for each experiment. Plates were screened at day three and day seven to assess the occurrence of bacterial lysis plaques formed by *A. polyphaga*.

2.34 Amoeba Co-culture (Predation)

For coculture experiments amoebae were harvested by gently tapping flasks, followed by centrifugation (300 rcf, 5 min) and washing twice in PAS, before numbers were adjusted to 2 x 10^5 cells per ml using a haemocytometer (Huws *et al.*, 2008). Cocultures were prepared in 24-well plates (Sarstedt®). Wells were seeded with *A. polyphaga* (2 x 10^5 cells per ml) and amoebae were allowed to adhere for 60 min before the addition of bacteria (2 x 10^8 cells per ml). Plates were incubated at 37^oC, and following various time intervals, wells were scraped, with dilution of the resulting cell suspension in saline (0.9% w/v) and colony counts of extracellular bacteria determined. Control bacterial and amoebal monocultures were also set up. Viability of *A. polyphaga* was assessed using the trypan blue exclusion assay (Gao et al., 1997).

2.35 RNA Extraction

Overnight cultures of the strains of interest were prepared in LB broth (with appropriate antibiotics). The following day the overnight cultures were diluted to an OD_{600} nm of 0.05 in 50ml of fresh LB broth (with appropriate antibiotics) in 200ml conical flasks that had been autoclaved a total of 3 times at 121^{o}C for 15 min. When the cultures had reached OD 0.2-0.3 10µM Isopropyl-b-D-thiogalactoside (IPTG) was added to induce expression of cloned gene for 30 min, at which time three 4ml samples were harvested. The RNA was stabilized using Qiagen RNA protect, 2 volumes of RNA protect was added to an RNase free tube before 1 volume of the culture was added and mixed vigorously. The mixture was then incubated for 5 min at room temperature before being spun in a centrifuge at 5,000rcf for 10 min. The supernatant was discarded and the pellet stored at -80^{o}C. Pellets were thawed before RNA extraction was performed following protocols #4 and #7 in the Qiagen RNeasy kit. In brief 15µl of proteinase K was added to 200µl of TE buffer containing lysozyme (30mM Tris.Cl, 1mM EDTA, pH8.0, containing 15mg/ml lysozyme) and used to resuspend the thawed pellet. The mixture was initially vortexed for 10 sec before being incubated at room temperature for 10 min, during which time the mixture was vortexed approximately every 2 min for 10 sec. Seven hundred µl of buffer RLT (with added β-mercaptoethanol) was then added to the mixture and vortexed vigorously. The mixture was then spun in a centrifuge at maximum speed for 2 min and the supernatant transferred to a fresh RNase free tube. 500µl of 96% ethanol was added to the supernatant and mixed via pipetting. Up to 700µl at a time was added to the RNeasy spin column with 2ml collection tube, which was spun using a centrifuge at 8,000rcf for 15s. The flow-through was discarded and 700µl of Buffer RW1 was added to the column, which was spun at 8,000rcf for 15s. Flow through was discarded. A further 500µl of Buffer RPE was added to the spin column, which was spun at 8,000rcf for 2 min. The column was transferred to a fresh collection tube and spun for a further 1 minu at 11,000rcf. Finally the column was transferred to an RNase free 1.5ml microcentrifuge tube; 30µl of RNase-free water was added to the column's membrane before spinning at 8,000rcf for 1 min to elute the RNA. As three 4ml samples were used for each strain and replicates, this step was repeated four times with a final volume of 120µl that was then stored at -80^{o}C or precipitate immediately by ethanol precipitation.

2.36 Precipitation of RNA

To ethanol precipitate the RNA samples, 5M Ammonium acetate was added to a final concentration of 0.5M and mixed well. Following this, 2.5 volumes of ice-cold ethanol was added, mixed and chilled at -20^{o}C for at least 20 min. RNA was recovered by centrifuging the samples for 15 min at maximum speed (11,000rcf) in a microcentrifuge at 4^{o}C if possible. The supernatant was removed carefully and the tube was re-spun for a few seconds at room temperature to remove any residual fluid. RNA pellets recovered in this manner do not require further drying. The RNA may be kept as an alcohol precipitate and stored at -80^{o}C or resuspended in 50µl sterile water.

A 2µl sample of the extracted RNA was run through a 1.5% agarose TBE gel to check the RNA. Once the presence of RNA had been confirmed the rest of the sample was treated with DNase (Ambion). Approximately 46µl of RNA was mixed with 1µl of DNase (2U/µl) and 5µl 10 x DNase buffer before incubating at 37^{o}C for 1 hour. DNase was inactivated by adding 5µl of DNase inactivation reagent to the tube which was mixed well before incubating at room temperature for 2 min. The tube's contents were mixed well again before spinning at maximum speed in a microcentrifuge for 2 min. The DNase inactivation reagent pelleted at the bottom of the tube enabling the RNA to be removed to a fresh centrifuge tube. After DNase treatment the RNA was again checked by running a sample through a 1.5% agarose TBE gel. A PCR was performed using a sample DNase treated RNA as template and *cpxP* primers cpxP Fw(XhoI) and cpxP Rv(BamHI) to confirm the absence of DNA.

2.37 Transmission electron microscope (TEM)

Cross sections of C2Bbe1 epithelial cells were prepared as follows. The cells were fixed in 0.165 molar phosphate buffered fixative, 2.0% glutaraldehyde, 2.5% paraformaldehyde for 90 min and buffer washed in 0.165 molar phosphate buffer for 30 min. Secondary fixation occurred with 0.165 molar phosphate buffered 2% Osmium tetroxide 90 min. After dehydration in a graded series of ethanol, the samples were embedded in polymerised araldite and polymerised at 50^{o}C for 48 h. Araldite embedded specimens were sectioned using a Reichert-Jung Ultracut E ultramicrotome. Samples were then stained with Uranlyl Acetate and Lead Citrate for photography in the transmission electron microscope. Sections were examined in

a Jeol JEM-2000FXII Transmission electron microscope and photographed using a Mega View Soft Imaging System digital camera. Calibration grid to determine real as distinct magnification: Agar Scientific cross grating, 2160 lines/mm. = 2159 intervals per 1000 microns for average spacing between lines. Specimen micrographs can only be correlated with calibration micrographs of the same magnification (and ideally from the same TEM Session)

2.38 Statistical analysis

All statistical analysis, except that performed on the transcriptome data, was performed using SigmaPlot 10.0 software. Each experiment was performed in triplicate unless otherwise indicated. For analysis of the significance of differences in adhesion and invasion levels, gfp expression, cytokine production and uptake and survival within J774-A1 macrophages, Student's t-test was used for comparison of two groups of data. A result was considered to be significant when $p<0.05$. For the microarray analysis, statistical significance was evaluated using one-way ANOVA and p-values were adjusted for multiple comparisons using the Benjamini and Hochberg method.

Chapter 3.0: Distribution of AIEC phenotype within *Escherichia coli* genus

3.1 Introduction

As previously stated, *Escherichia coli*, is ubiquitous and a usually harmless commensal of the human GIT flora as well as a common member of the flora of other mammals and birds. Furthermore, *E. coli* also include pathogens which are an important cause of sickness and mortality throughout the world (Souza *et al.*, 1999). Virulence is often associated with distinct clusters of genes referred to as 'pathogenicity islands (PAIs)' (Wirth *et al.*, 2006). PAIs are present on the genomes of pathogenic strains but absent from the genomes of non-pathogenic members of the same or related species (Hacker & Kaper, 2000). The effects of virulence on the patterns of evolution of the *E. coli* genome remain unclear (Wirth *et al.*, 2006). Previous studies have shown that natural populations of *E. coli* harbor an extensive genetic diversity that is organized in a limited number of clones (Guttman & Dykhuizen, 1994; Pupo & Richardson, 1995; Souza *et al.*, 1999; Whittam, 1989). The first attempt to examine the genetic structure and diversity of *E. coli*, *known as the Milkman study*, used multilocus enzyme electrophoresis (MLEE) to analyse 849 isolates obtained primarily from humans (Milkman, 1973). MLEE uses the relative electrophoretic mobilities (EM) of multiple intracellular metabolic enzymes to characterise and differentiate organisms by generating an electromorph type (ET). The relatedness of isolates can then be visualized from the differences between the ETs (Stanley & Wilson, 2003). This study led to MLEE being adopted as a common technique for the study of bacterial diversity. Over a number of years further studies using MLEE with more extensive strain collections claimed that certain combinations of alleles occurred multiple times, indicating a clonal population structure with recombination serving a limited role in the evolution of *E. coli* (Ochman *et al.*, 1983; Ochman & Selander, 1984b; Selander & Levin, 1980; Wirth *et al.*, 2006). However, more recent studies have shown strong evidence for recombination playing a significant role in shaping the genomic evolution and diversification of *E. coli* (Cooper, 2007; Didelot *et al.*, 2012; Mau *et al.*, 2006; Winkler & Kao, 2012). Recombination can influence the evolution of *E. coli* both by replacing fragments of genes with an homologous sequence and also by

introducing new genes (Didelot *et al.*, 2012). In one study comparing 27 whole genomes of *E. coli* several non-random patterns of these events were identified which correlated with important changes in the lifestyle of the bacteria (Didelot *et al.*, 2012). In particular, the genomes of three enterohaemorrhagic (EHEC) strains have converged from originally separate backgrounds as a result of both homologous and non-homologous recombination (Didelot *et al.*, 2012). Furthermore, intraspecific recombination in *E. coli* has been shown to be much more common than previously appreciated and may even show a bias for certain types of genes, including those involved in recombination, transport and motility (Mau *et al.*, 2006). Construction of a multiple whole genome alignment facilitates a global survey of recombination among published genome sequences of several *E. coli* (Mau *et al.*, 2006) and as additional *E. coli* strains are sequenced, the role of homologous recombination in bacterial genome evolution will become clearer, and may force reassessment of traditional methods for describing relationships among bacterial taxa (Mau *et al.*, 2006).

3.1.1 The ECOR strain collection

The *E. coli* reference collection, referred to as ECOR, is a standard collection of 72 wildtype strains that are representative of the known genetic diversity of the species as indicated by MLEE (see fig. 3.1) (Ochman & Selander, 1984a). These 72 *E. coli* strains were isolated from humans and 16 other mammalian hosts incorporating both geographical distribution and varied host range. Although both commensal and pathogenic strains (mainly UPEC) were included, the collection does not contain diarrhoeagenic strains (Chaudhuri & Henderson, 2012). To facilitate the study of diarrhoeagenic *E. coli*, a separate collection of 78 diarrhoeagenic *E. coli* (DEC) strains has been established, again chosen to represent the diversity seen using MLEE (Chaudhuri & Henderson, 2012; Whittam *et al.*, 1993).

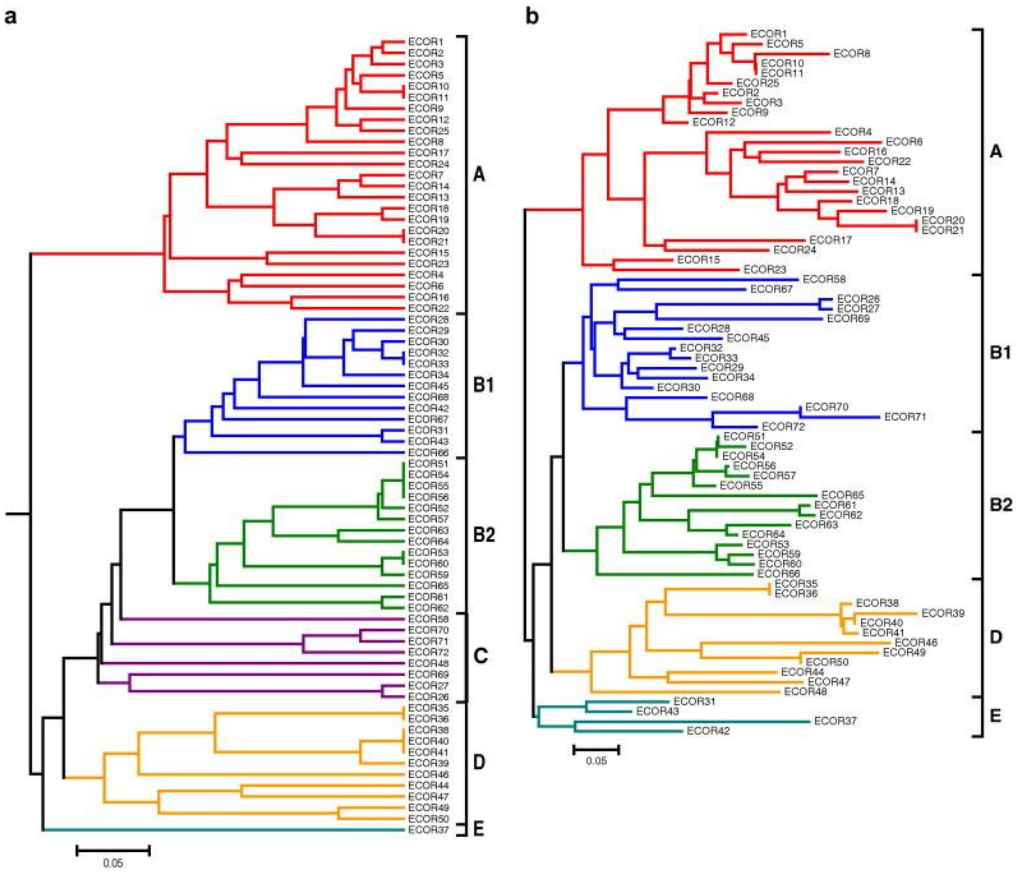


Figure 3.1: Phylogenetic relationships of the ECOR 72 collection assessed by multi-locus enzyme electrophoresis (MLEE). A) Phylogeny constructed using the UPGMA method B) Phylogeny constructed using the neighbour-joining method (Chaudhuri and Henderson 2012).

MLEE revealed four main phylogenetic groups designated A, B1, B2 and D, plus a minor group E although this has been largely ignored as it has been shown to cluster inconsistently in subsequent analyse (Wirth *et al.*, 2006). More recent data suggests that group A, which includes *E. coli* K-12, was the earliest to diverge, with groups B1 and B2 as sister-taxa (Chaudhuri & Henderson, 2012). Independently of MLEE, the major phylogenetic groups have been consistently supported in a variety of studies utilising various data types including randomly amplified polymorphic DNA (RAPD) (Desjardins *et al.*, 1995), multiple-locus variable-number tandem repeats analysis (MLVA) (Lindstedt *et al.*, 2007) and variation at mononucleotide repeat loci (Diamant *et al.*, 2004). A drawback of MLEE is that enzymes with little sequence similarity may exhibit similar electrophoretic mobility (Chaudhuri & Henderson, 2012) therefore multilocus sequence typing (MLST) was proposed as an alternative to MLEE to examine differences between bacterial strains (Maiden *et al.*, 1998). MLST measures the DNA sequence variations in a set of housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) and characterizes strains by their unique allelic profiles (Wirth *et al.*, 2006). A number of studies have utilised MLST to undertake large scale investigations of *E. coli* (Reid *et al.*, 2000; Turner *et al.*, 2006; Wirth *et al.*, 2006). Such studies have suggested that the diversity of *E. coli* may extend beyond the range represented in the ECOR collection. Moreover, a recent MLST study investigated a number of isolates that were phenotypically indistinguishable from *E. coli* but exhibited divergent nucleotide sequences (Walk *et al.*, 2009) . Such strains were more likely to be found in environmental isolates than the mammalian gastrointestinal tract, suggesting they may represent environmentally adapted organisms (Chaudhuri & Henderson, 2012). Recent studies including several genomic methods like DNA microarrays, optical mapping, and whole genome sequencing have shed new light on the level of diversity within bacterial populations, making it clear that the level of genomic diversity had been grossly underestimated previously (Jackson *et al.*, 2007; Jackson *et al.*, 2011). DNA microarrays are becoming increasingly useful tools to investigate diversity, for example one study utilised microarray technology to examine the gene content of 207 diverse isolates of *E. coli* and *Shigella* spp. as well as the entire ECOR collection (Jackson *et al*., 2011). Interestingly, for the most part, the whole genome analysis of the ECOR collection correlated well with previous designated phylogenetic groups as shown by MLST. However, a number of notable exceptions were identified. For example,

strains ECOR70, ECOR71, and ECOR72 that were previously defined in group B1 were found to be part of group A and notably, as with MLST, array analysis showed group D strains to be more heterogeneous than other groupings, seemingly reflecting diverse and distinct lineages of Group D strains (Jackson *et al.*, 2011). It is important to note that the diversity within the species *E. coli*, and the overlap in gene content between this and related species is far greater than many had anticipated and represents a broad set of functions for adapting to many different environments (Lukjancenko *et al.*, 2010).

3.1.2 Diversity among AIEC

Most commensal strains tend to belong to phylogenetic groups A or B1, whilst extraintestinal pathogenic strains typically belong to phylogenetic groups B2 or D, and they possess more virulence factors than commensal strains which allow them to induce diseases in both healthy and compromised hosts (Abdallah *et al.*, 2011; Lee *et al.*, 2010). Over the last thirty years, it has been becoming increasingly evident that groups B2 and D are emerging as the dominant phylogroups in the western world (Kotlowski *et al.*, 2007). Moreover, there is increasing evidence that IBD isolates tend to be members of the B2 or D groups and this has been suggested to correlate with the increased frequency of IBD in the western world (Kotlowski *et al.*, 2007). The AIEC strains isolated to date are diverse and belong to distinct serotypes (Martinez-Medina *et al.*, 2009c). Moreover, even though AIEC are primarily associated with group B2, AIEC strains belonging to the A, B1, and D phylogroups have also been isolated (Martinez-Medina *et al.*, 2009a; Martinez-Medina *et al.*, 2009c). As previously stated, AIEC display ExPEC features and harbour virulence associated genes characteristic of ExPEC. A high diversity of serotypes and virulence gene profiles exists among ExPEC strains, which complicates their classification into pathotypes and similarly a variety of seropathotypes can also be found among AIEC strains (Martinez-Medina *et al.*, 2009c). A comparison of two AIEC genome sequences showed a close relationship between these two strains (LF82and NRG685c) and suggested AIEC-specific gene sets (Nash *et al.*, 2010); however comparisons with a third more divergent AIEC strain, HM605 highlighted that the similarity between the two previous strains is only indicative of their serotype suggesting there are no AIEC specific genes (Chaudhuri & Henderson,

2012; Clarke *et al.*, 2011). Moreover, in a recent study several AIEC isolates belonging to the same clonal group showed different invasive properties (Martinez-Medina *et al.*, 2009a) suggesting that the AIEC pathovar harbors great internal variability and genomic diversity possibly due to differential expression of key genes or specific pathoadaptive mutations (Chaudhuri & Henderson, 2012). The lack of conserved genes in AIEC also suggests no pathogenicity islands and the possible role of recombination in the diversity of this pathotype.

3.2 Objectives

The main characteristics for inclusion into AIEC are (i) the ability to colonise the intestinal mucosa by adhering to and invading intestinal epithelial cells (ii) ability to survive and to replicate within macrophages and (iii) to induce the release of high amounts of pro-inflammatory cytokines such as TNF-α and IL1-β (Boudeau *et al.*, 1999; Boudeau *et al.*, 2001; Darfeuille-Michaud, 2002; Darfeuille-Michaud, 2004; Martin *et al.*, 2004). Therefore, the initial aim of this research was to examine replication in macrophages to determine if this phenotype is widespread in the *E. coli* genus. To date, little is known whether this characteristic is common to a large number of *E. coli*. To achieve this objective, we determined the ability of the ECOR72 collection and 12 other wildtype *E coli* strains to survive and replicate within macrophages.

3.3 Results

3.3.1 The ability to replicate in J774.A1 macrophages is distributed across all phylogenetic groups.

An important feature of AIEC is its ability to persist and replicate in macrophages. In order to determine whether the ability to replicate within macrophages is widespread throughout the *E. coli* genus or restricted to a particular phylogroup or clade it was decided to use the well-characterised ECOR72 library and the macrophage-like J774A.1 cell line (ATCC TIB-67). The experimental set up consisted of 3 biological replicates and for each strain. The *E. coli* strains were grown overnight, adjusted to an OD600nm=1 and added to $4x10^5$ macrophages at a multiplicity of infection of 10. After 2 hours infection gentamycin was added to kill any extracellular bacteria and macrophage were lysed to determine the level of bacteria within the macrophage at

1h and 6h post-gentamicin. The level of bacterial replication was determined by comparing 1h post-gentamicin with 6h post-gentamicin. The results of the intramacrophage replication assays are summarised in figure 3.2. A fold replication of 1 was designated to indicate the net growth/death of the different *E. coli* strains over the 6 hours. The known AIEC strain HM605 survived and replicated within macrophages over this period whilst only a few strains showed a significant decrease in numbers over the 6 hours e.g. ECOR-1, ECOR-32 and ECOR-63. This result implied that intramacrophage persistence is not a unique feature of AIEC; on the contrary it appears that the ability of *E. coli* to persist is widespread across the phylogenetic groups A, B1, B2 and D.

The mean number of replication was 2-fold range and any strain that had the ability to replicate ≥1 standard deviation from this mean was considered to replicate within the macrophage. Initially, 10 ECOR strains showed high levels of replication. However, secondary screening of these strains showed that only 5 out of the 10 ECOR strains showed reproducibly similar levels of intramacrophage replication as AIEC HM605 (see fig. 3.3). Therefore these strains could potentially be characterised as AIEC due to their ability to replicate at high levels similar to AIEC strain HM605. These strains were again distributed across each phylogenetic group. The results of the intramacrophage replication assays support the conclusion that AIEC are a diverse group and are not a clonal group.

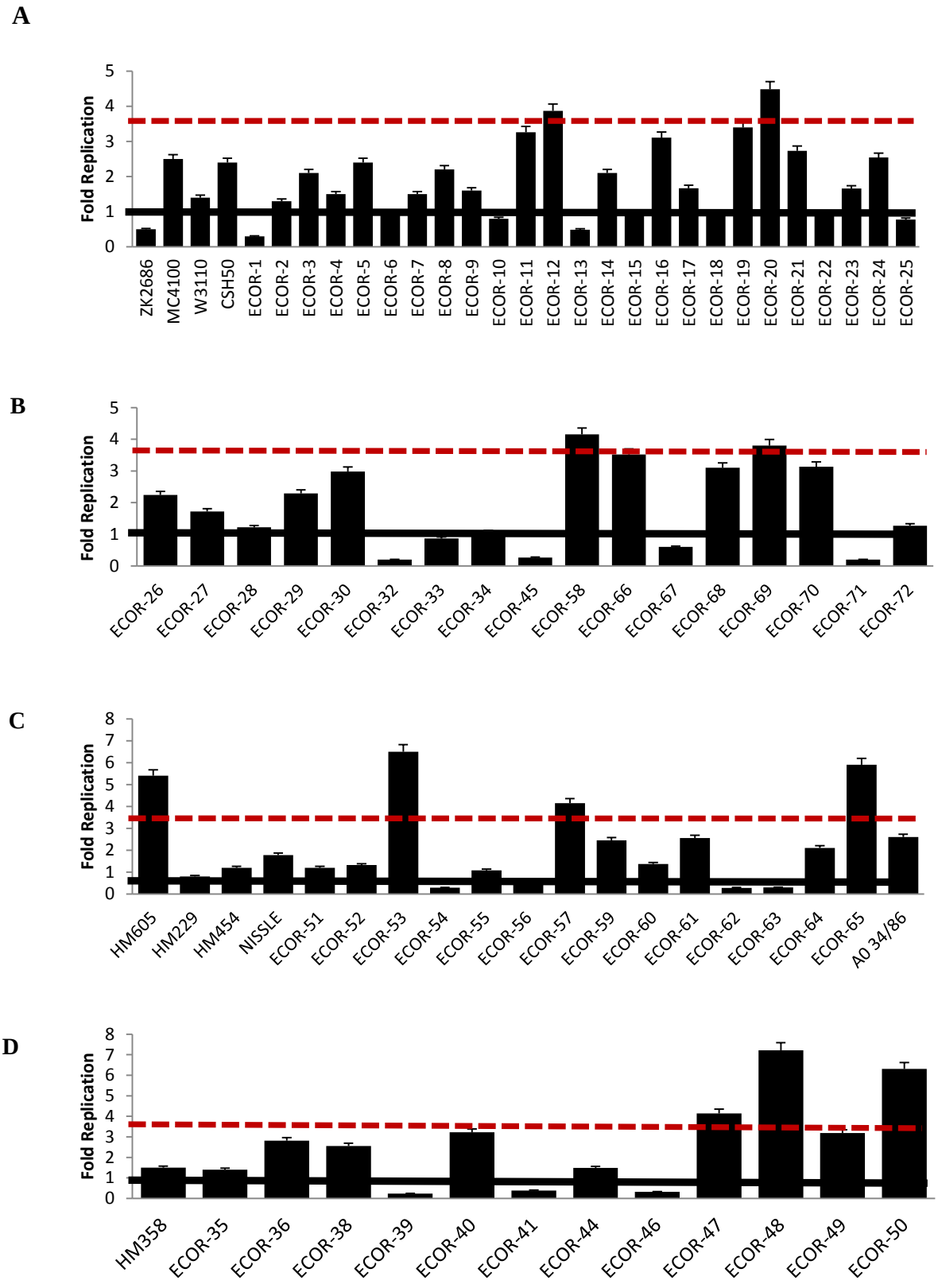


Figure 3.2: Distribution of intramacrophage replicating *Escherichia coli* within each phylogenetic group. A) Group A, B) Group B1, C) Group B2 and D) Group D. Black line refers to the net growth/death of the *E. coli* strains over the 6 hours. Red dotted line represents the value of 1 standard deviation greater than the average 2-fold replication (n=3.6). Results shown are from 3 experiments with 1 technical replicate per experiment.

3.3.1.1 A number of ECOR strains showed intramacrophage replication levels similar to known AIEC strain HM605.

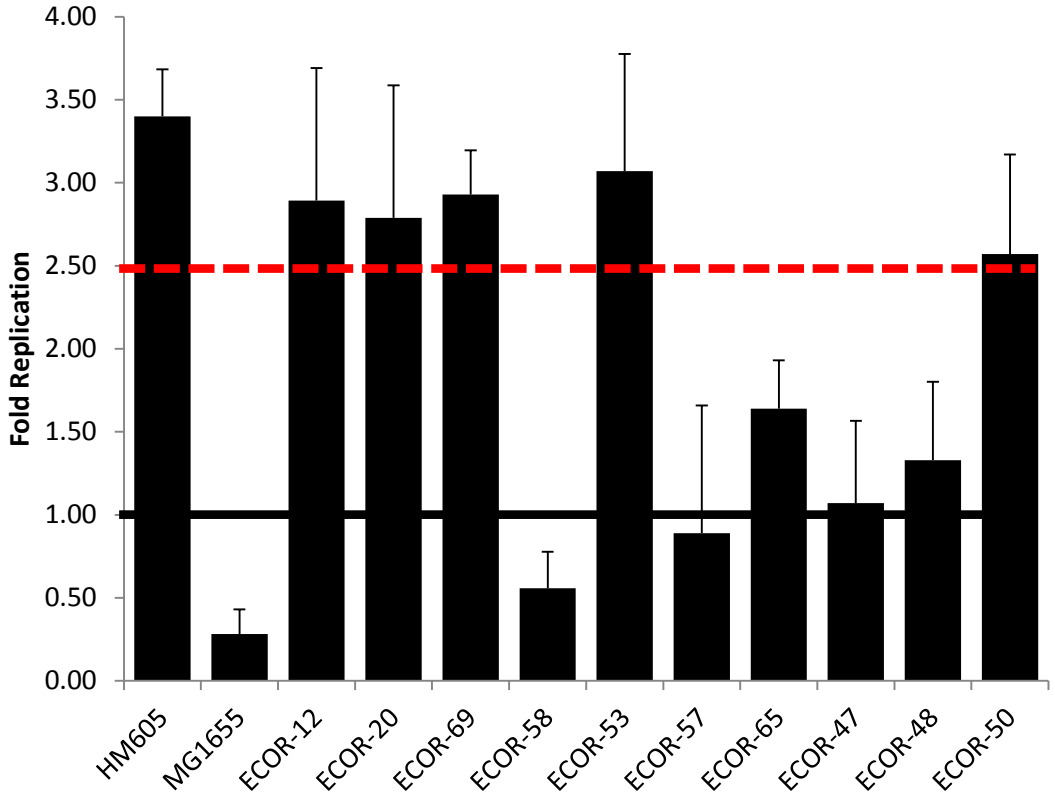


Figure 3.3: Fold replication of the ECOR strains showing similar levels as known AIEC strain HM605 in J774.A1 macrophage. Five strains showed reproducibly similar levels of intramacrophage replication as AIEC HM605. Black line refers to the net growth/death (n=1) of the *E. coli* strains over the 6 hours. Red dotted line represents the value of 1 standard deviation greater than the average fold replication (n=2.5). Results shown are from 3 experiments with 1 technical replicate per experiment.

3.3.2 Adhesion and invasion of C2Bbe1 epithelial cells

The next stage in assessing the distribution of the AIEC phenotype across the *E. coli* genus was to examine the ECOR strains identified as capable of intramacrophage replication for their ability to adhere to, and invade, C2Bbe1 (ATCC **CRL-2102)** colonic epithelial cells. This cell line is a clone of the better known Caco-2 and is used because C2BBe1 cells form a polarized monolayer with an apical brush border (BB) morphologically comparable to that of the human colon. To test for an effect on adherence and invasion, bacteria were added to C2Bbe1 epithelial cells at a multiplicity of infection of 10 bacteria per epithelial cell and allowed to infect for 3 hours. Following infection, the monolayers of one plate were washed twice in sterile 1X PBS, lysed with 1% triton X-100, serially diluted and plated onto LB plates to determine the number of colony forming units (cfu) corresponding to the total number of cell associated bacteria (adherent and intracellular bacteria). Adhesion ability is calculated as the mean number of bacteria per C2Bbe1 cell after 3 hours (Martinez-Medina *et al.*, 2009c). For the bacterial invasion assays, the number of intracellular bacteria was determined by plating following 1 hour gentamicin (100μg/ml) treatment and after cell lysis with 1% Triton X-100. Invasion ability is calculated as the percentage of the original inoculum that survives after 1 hour gentamicin treatment. Strains with an I_ADH ≥ 1.0% and an I_INV ≥ 0.1% can be classified as AIEC (Martinez-Medina *et al.*, 2009c). The bacterial adhesion results demonstrated that all ECOR strains examined, apart from ECOR-50, could adhere to C2Bbe1 epithelial cells (see Table 3.1). Similarly, all of the ECOR strains examined, apart from ECOR-50, were able to invade C2Bbe1 epithelial cells (see Table 3.1). Therefore, after determining the capacity of the ECOR strains to adhere to and invade intestinal epithelial cells and their ability to survive and replicate within macrophages, we classified 4 strains out of 84 tested (i.e. 4.96%) as possessing AIEC characteristics.

Strain	Adhesion ability (I_ADH)	Invasive ability (I_INV)
HM605 (AIEC)	3.12 ± 1.25	0.22 ± 0.03
ECOR-12	6.75 ± 1.34	0.42 ± 0.18
ECOR-20	6.35 ± 2.41	0.73 ± 0.20
ECOR-69	1.77 ± 0.51	0.20 ± 0.10
ECOR-53	3.83 ± 0.19	0.12 ± 0.03
ECOR-50	0.05 ± 0.09	0.02 ± 0.01

Table 3.1: Adhesion and invasion of C2Bbe1 epithelial cells. Adhesion ability is calculated as the mean number of bacteria per C2Bbe1 cell after 3 hours. Invasion ability is calculated as the percentage of inoculum surviving after 1 hour gentamicin treatment. Those strains with an I_ADH ≥ 1.0 and an I_INV ≥ 0.1% were classified as adherent and invasive strains. All strains, apart from ECOR-50, were able to both adhere to and invade C2BBe1 epithelial cells. Results are from triplicate determinations.

3.3.3 Investigating the role of low pH on intracellular replication of ECOR strains

The strategy used by AIEC to resist macrophage degradation is unknown. However, it has been reported that the AIEC strain LF82 is able to survive and to replicate within a compartment harbouring hostile features of mature phagolysosomes including acidic pH and active cathepsin D (Bringer *et al.*, 2006). Moreover, the acidification of then phagolysosome has been reported as necessary for the replication of AIEC LF82 bacteria within J774 macrophages (Bringer *et al.*, 2006). In order to investigate if acidification of the phagosome is required for intracellular replication of the four ECOR strains identified in this study as having AIEC characteristics, the effect of the vacuolar pH-neutralizing reagent ammonium chloride (NH_4Cl) on the replication ability within J774 macrophages was examined. Ammonium chloride (NH_4Cl) is a lysosomotropic compound that is membrane permeable in its non-protonated forms but impermeable when protonated in acidic phagosomes (Bringer *et al.*, 2006). The result of this is that the pH of the phagosome

is neutralised. NH_4Cl was not directly lethal to either the bacteria or the host cells at the concentrations used. Following treatment with NH_4Cl, the results indicate that the intracellular replication of some strains, such as HM605 and ECOR-69, does require acidification of the phagosome, whereas the replication of strains ECOR-20 and ECOR-53 appears to be increased in the presence of NH_4Cl, indicating that acidification of the phagosome may compromise the intracellular replication of these strains (See fig 3.4). The replication of the final strain, ECOR-12, appeared to be independent of NH_4Cl. This data suggest that an acidic environment is not required for intracellular replication of all strains examined and further highlights the diversity associated with the AIEC phenotype.

In an extension of the aforementioned phagosome acidification assay, growth curves in low pH minimal medium were conducted to determine if pH dependent intracellular replication corresponds to an increased level of acid tolerance. For these tests a pH of 4.5 was chosen as this is generally considered to be the intracellular pH of the mature phagosome (Jabado *et al.*, 2000). Growth curves for AIEC strains HM605, LF82 and two K-12 strains ZK2686 and MG1655 are presented in Figure 3.5. From the results of the growth experiments it can be seen that both AIEC strains appear to have an increased tolerance to low pH compared to the two *E. coli* K-12 strains. The determination of the growth rates of the 4 ECOR strains identified in this study as having the AIEC phenotype revealed that AIEC strain HM605 and ECOR-69, strains that both require acidification of the phagosome (pH dependent) for intracellular replication, show similar growth rates at low pH. Both strains show significantly higher growth rates at pH 4.5 compared to MG1655 (a K-12 strain non able to replicate in the macrophage) and ECOR-20 and ECOR-53, strains whose intramacrophage replication is inhibited by the low pH of the phagosome. Together these findings indicate possibly 3 different types of AIEC intramacrophage replication. Replication dependent on the acidification of the phagosome (HM605 and ECOR-69) similar to AIEC LF82, increased replication when the acidification of the phagosome is neutralised (ECOR-12 and ECOR-69) and replication independent of both the acidification and neutralisation of the phagosome (ECOR-12). However, as all strains can undergo intramacrophage replication these experiments indicate that phagosome acidification and tolerance to low pH are not prerequisites for the intracellular replication of *E. coli*.

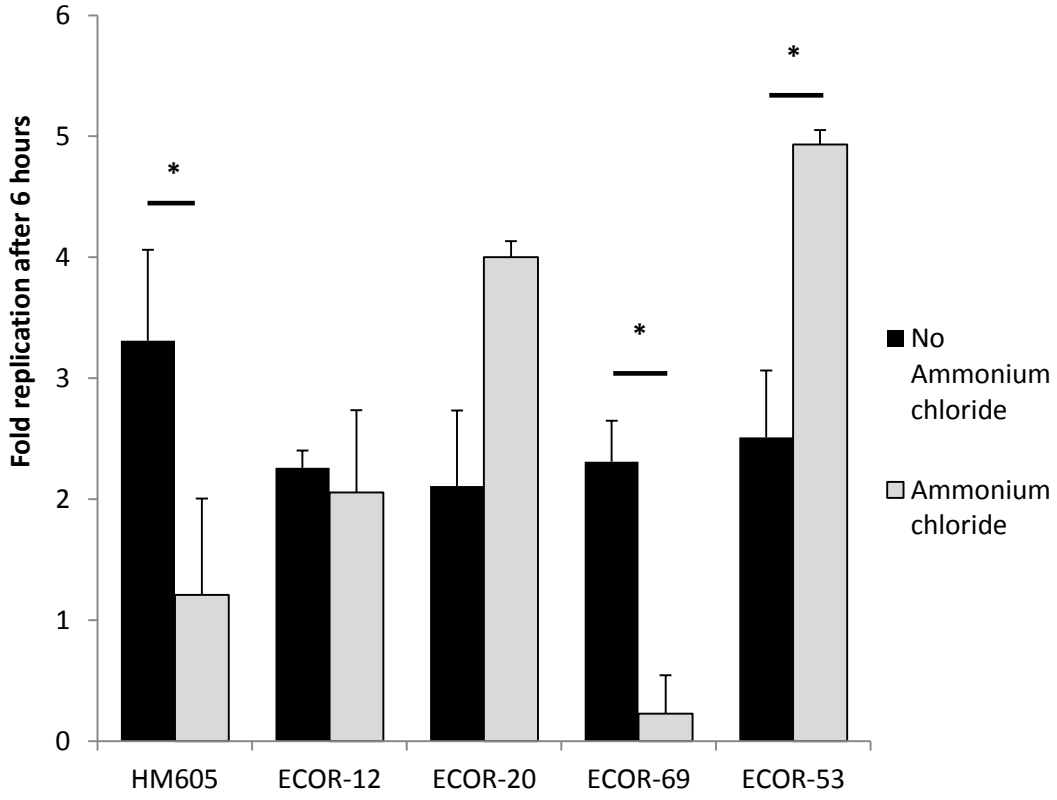


Figure 3.4: Treatment of infected macrophage with 2mM NH_4Cl to neutralise phagosome. Results are expressed as the number of viable bacteria at 6 h post-gentamicin relative to that obtained at 1h post-gentamicin, taken as 100%. Results show some strains ability to persist is pH dependent as they require acidification of the phagosome such as HM605 and ECOR-69, whereas strains such as ECOR-20 and ECOR-53 ability to persist appear to be pH independent. Results are from triplicate determinations. For analysis of the significance of differences in uptake and survival within J774-A1 macrophages, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

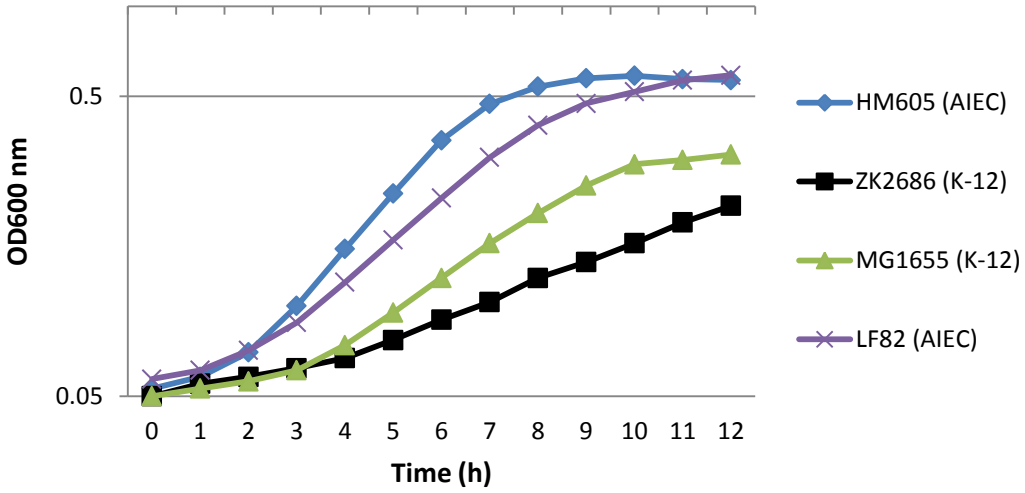


Fig 3.5: Growth in M9 minimal media (pH 4.5) supplemented with 0.02% (w/v) glucose and buffered with 100mM MES. The data represent the mean results of 3 experiments each one carried out in triplicate. Overnight cultures were diluted in fresh media to OD_{600}=0.05 and 150µl of this diluted culture was inoculated, in triplicate, into wells of a 96 well optical flat bottom plate. OD_{600} readings were taken every 15 minutes over a period of 12 hours using a MWG Sirius HT plate reader, programmed to incubate plates at 37°C. Only the values for every 60 minutes are plotted here.

Strain	Growth rate (h^{-1})
HM605 (AIEC)	0.098
MG1655 (K-12)	0.031
ECOR-12	0.068
ECOR-20	0.046
ECOR-69	0.083
ECOR-53	0.056

Table 3.3: Growth rates of *E. coli* strains in pH 4.5 M9 minimal media supplemented with 0.02% glucose and 100mM MES. AIEC strain HM605 and ECOR-69 who both require acidification of the phagosome (pH dependent) show similar growth rates at low pH. Both strains show higher growth rates compared to both ECOR-20 and ECOR-53.

3.3.4 Detection of cytokines TNF-α and IL1-β in the supernatant of infected macrophages

It has been previously reported that AIEC LF82-infected macrophage secrete large amounts of tumour necrosis factor-alpha (TNF-α), which in turn increases the intramacrophagic replication of LF82 (Bringer *et al.*, 2011; Glasser *et al.*, 2001). Similarly, in the same studies it was reported that no interleukin-1 beta (IL1-β) production could be detected 1 h post LF82 infection of macrophages pre-stimulated with LPS overnight (Glasser *et al.*, 2001). In order to explore the possible mechanisms in an inflammatory response induced by the ECOR strains, the levels of cytokines TNF-α and IL1-β produced by these strains was analysed using Meso Scale Discovery technology (MSD® Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions.

Quantification of TNF-α secretion was completed with J774.A1 macrophage infected with bacteria at an MOI of 10. After 1h post-gentamicin treatment, similar amounts of TNF-α were induced by all *E. coli* strains (see fig 3.6 A). At 24h post-gentamicin, all of the *E. coli* strains examined, including the commensal strain MG1655, showed an increase in TNF-α induction. No significant difference was observed between MG1655 and HM605 or any of the AIEC-like ECOR strains (see fig 3.6 A). This suggests that, in contrast to what was previously published, the production of large amounts of TNF-α is not a distinguishing feature of AIEC, as similar amounts of TNF-α are induced by a known AIEC strain and a commensal control. The quantification of IL1-β release was obtained again with J774.A1 macrophage infected for 2 h with bacteria at an MOI of 10. After 1h post-gentamicin treatment, very low levels of IL-1b could be detected from all of the infected macrophage (see fig 3.6 B). However, at 24 h post-gentamicin, macrophage infected with HM605 and the AIEC-like ECOR strains showed release of significant quantities of IL1-β (see fig 3.6 B). In contrast, a much reduced level of IL1-β was detected in macrophage infected with the MG1655 control. These findings indicate that AIEC can induce higher amounts of IL1-β compared to a non-replicating *E. coli* strain.

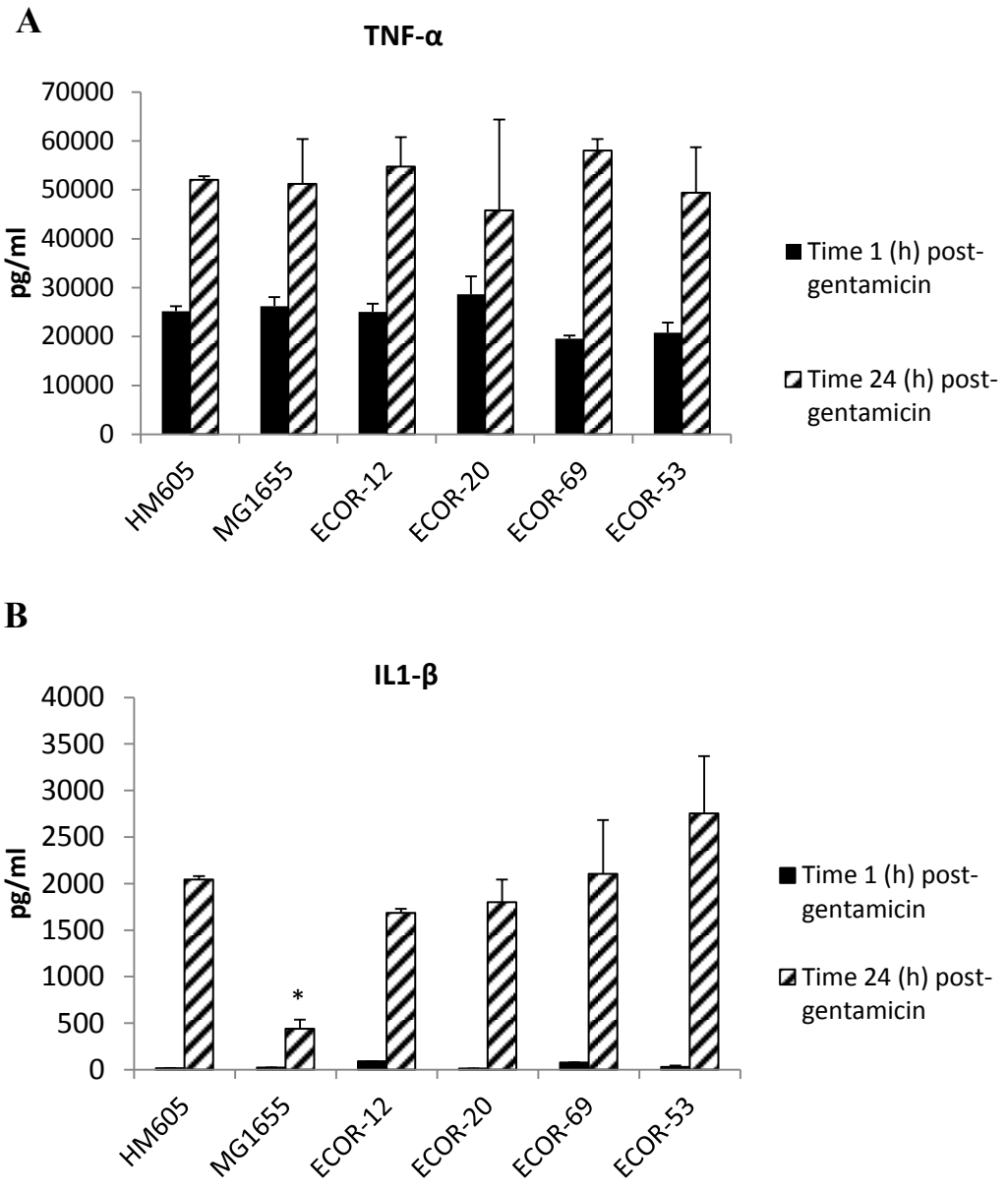


Fig 3.6: Production of proinflammatory cytokines TNF-α and IL1-β by J774-A1 cells after *E. coli* infection. A) Production of pro-inflammatory cytokine TNF-α by J774.A1 cells after infection. TNF- α does not appear to be induced to significantly high levels as all strains induce similar amounts of TNF- α. B) Production of pro-inflammatory cytokine IL1-β by J774.A1 cells after infection. High levels of IL1-β was induced by all strains, apart from the K-12 strain MG1655. Results are from triplicate determinations. For analysis of the significance of differences in cytokine production, Student's t-test was used for comparison of two groups of data (∗ P-value ≤ 0.05).

Time 1 (h) post-gentamicin

Time 24 (h) post-gentamicin

Time 1 (h)

3.3.5 Amoeba as evolutionary reservoirs for intracellular bacterial pathogens

We have established that the ability to replicate in macrophage is a phenotype that is widespread throughout the *E. coli* genus. However, what could be the selection for the evolution of this phenotype? The coincidental evolution hypothesis suggests that virulence traits in bacteria might result from selective pressure exerted by protozoan predators such as amoeba. Thus, grazing resistance may be an evolutionarily exaptation for bacterial pathogenicity (Adiba *et al.*, 2010). A number of microorganisms such as *Legionella pneumophila* (Atlan *et al.*, 2012; Rowbotham, 1986), *E. coli* O157 (Barker *et al.*, 1999) and *Mycobacterium* spp (Salah *et al.*, 2009) have developed mechanisms to become resistant to killing by amoebae since they are not internalized or are able to survive, replicate and exit free-living amoebae after internalization (Wildschutte & Lawrence, 2007). These genetic adaptive changes include evolution by beneficial mutations and by the lateral transfer of genes (Ochman *et al.*, 2000) that led to the expression of symbiotic or pathogenic phenotypes according to their impact on the host cell (Greub & Raoult, 2004). Consequently, amoebae represent a potent evolutionary reservoir for internalized microbes. The aim was to determine if the intramacrophage replicating ECOR strains were resistant to killing and/or grazing by predatory amoeba.

Table 3.1 Examples of bacteria that have evolved resistance to killing by amoeba (modified from Greub, G., and Raoult, D. 2004)

Bacteria resistant to amoeba killing	Amoeba Host	Life style	Ref.
Legionella pneumophila	Many species	Facultative intracellular	(Rowbotham, 1980)
E. coli O157	*Acanthamoeba*	Extracellular	(Barker *et al.*, 1999)
Helicobacter pylori	*Acanthamoeba*	Facultative intracellular	(Winiecka-Krusnell *et al.*, 2002)
Listeria monocytogenes	*Acanthamoeba*	Facultative intracellular	(Ly & Muller, 1990)
Vibrio cholerae	*Acanthamoeba, Naegleria*	Extracellular	(Thom *et al.*, 1992)
Francisella tularensis	*Acanthamoeba*	Facultative intracellular	(Berdal *et al.*, 1996)
Coxiella burnetii	*Acanthamoeba*	Obligate intracellular	(La Scola & Raoult, 2001)
Mycobacterium avium	*Acanthamoeba*	Facultative intracellular	(Cirillo *et al.*, 1997; Miltner & Bermudez, 2000)
Mycobacterium leprae	*Acanthamoeba*	Facultative intracellular	(Lahiri & Krahenbuhl, 2008; Vaerewijck *et al.*, 2005)
Mycobacterium marinum	*Acanthamoeba*	Facultative intracellular	(Cirillo *et al.*, 1997)

3.3.5.1 AIEC HM605 is resistant to killing by amoeba

In order to compare the ability of HM605 to resist grazing by predatory protozoa with that of a commensal strain, a plating assay was performed for each strain with a bacterial population size of 10^8 cells and an *Acanthamoeba polyphaga* population

size of 10^2 cells (Adiba *et al.*, 2010). Therefore cells were mixed and spread onto the surface of agar plates. After several days at 25^{o}C the formation of plaques was examined. A strain of *E. coli* associated with neonatal meningitis, RS218, was used as a positive control as this strain has been previously shown to be resistant to killing by amoeba and undergo intramacrophage replication (Alsam *et al.*, 2006). The results (see fig 3.7) show that no plaques (indicative of grazing by the *A. polyphaga*) were observed for HM605 or the positive control RS218, suggesting that these strains have the grazing resistance phenotype. In contrast, plaques were observed with MG1655, suggesting that this strain is not able to resist grazing by the amoeba (see fig 3.7). These data suggest that there may be a correlation between grazing resistance and the ability to replicate inside macrophages.

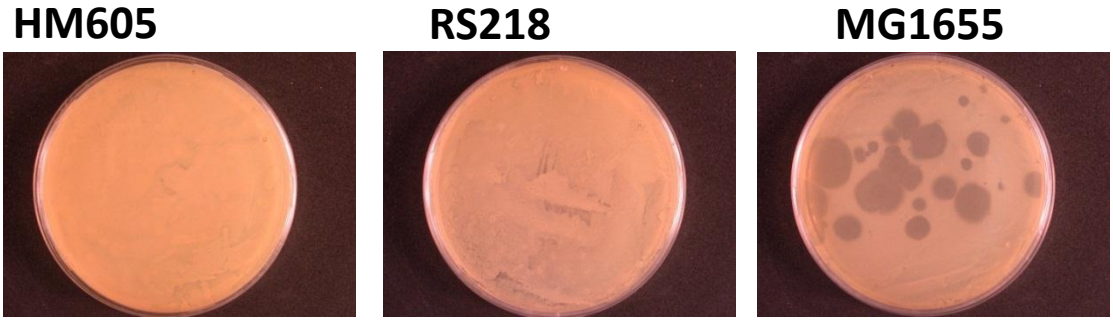


Fig 3.7: Amoeba grazing assay. AIEC strain HM605 and NMEC strain RS218 were plated with *Acanthamoeba polyphaga*. No bacterial lysis plaques were observed, characteristic of the grazing resistance phenotype. HM605 appears to be resistant to killing by amoeba. MG1655 K-12 was plated with *Acanthamoeba polyphaga*. Bacterial lysis plaques were observed, characteristic of grazing phenotype. Shown is the result of a typical experiment. Experiments were carried out in triplicate.

3.3.5.2 Amoeba grazing phenotype of the ECOR strains studied

As AIEC HM605 is resistant to killing by amoeba it was important to determine whether any of the AIEC-like ECOR strains also had the grazing resistance phenotype. Eight non-intramacrophage replicating ECOR strains (2 from each phylogenetic group) were also selected as controls to determine if there is a correlation between intramacrophage replication and grazing resistance. The results are summarised in Table 3.3.

Strain	Bacterial lysis plaques (+ refers to formation of plaques, -refers to no plaque formation)	Intramacrophage replication (+ refers to ability to replicate, - refers to inability to replicate	Amoeba
HM605 (AIEC)	– – –	+	GR
RS218 (NMEC)	– – –	+	GR
MG1655 (K-12)	+ + +	-	G
ECOR-12	**– – –**	**+**	**GR**
ECOR-20	**– + +**	**+**	**G**
ECOR-69	**– – –**	**+**	**GR**
ECOR-53	**– – –**	**+**	**GR**
ECOR-1 (A)	+ + +	-	G
ECOR-13 (A)	– + +	-	G
ECOR-32 (B1)	– + +	-	G
ECOR-45 (B1)	+ + +	-	G
ECOR-54 (B2)	– – –	-	GR
ECOR-56 (B2)	+ + +	-	G
ECOR-39 (D)	+ + +	-	G
ECOR-46 (D)	+ + +	-	G

Table 3.3: Amoeba grazing phenotype of the *E. coli* strains studied. Strains in red are ECOR strains showing AIEC like characteristics. *E. coli* strains with the ability to survive inside macrophage shows higher grazing resistance suggesting a correlation between intramacrophage survival and resistance to killing by amoeba. Results are from triplicate determinations. **G= Grazing, GR= Grazing Resistance.**

The ‘‘grazing resistance’’ (GR) phenotype corresponds to the plates where we did not observe any plaques in the three replicates. The ‘‘grazing’’ (G) phenotype corresponds to plates where we observed lysis plaques for at least one replicate or for all three replicates (Adiba *et al.*, 2010). The results revealed that approximately 75% (3/4) of the intramacrophage replicating ECOR strains show grazing resistance phenotype similar to HM605 (see table 3.3 and see fig 3.7). In contrast, only 12.5% (1/8) of non-intramacrophage replicating ECOR strains showed grazing resistance phenotype. The combined results from these experiments indicate a possible correlation between intramacrophage replication and resistance to killing by amoeba as significantly more intramacrophage replicating strains showed grazing resistance phenotype (Student t-test, P value ≤ 0.05).

3.3.6 Co-interactions of bacteria and amoeba

In order to determine if amoeba were capable of sustaining bacterial growth or if the bacteria were killing the amoeba, co-culture/predation assays were conducted as previously described (Huws *et al.*, 2008). In the co-culture system, the extracellular bacterial viable count was compared with the survival of bacteria grown without amoebae. If the comparative counts in the presence of amoebae were lower than this was evidence of predation. Similarly, if counts were higher than amoebae were sustaining bacterial growth and/or survival. Co-culture/ predation assays for HM605, RS218 and MG1655 are presented in Figure 3.8. It can be seen that at T0 (no significant difference is observed as similar numbers are seen in all strains (fig 3.8). However, 24 hours post-infection MG1655 was significantly ($P < 0.05$) lower in numbers in the presence of the amoebae compared with controls in the absence of amoebae. This indicates the occurrence of predation suggesting that the amoebae are feeding on MG1655 (fig 3.8 B). In contrast, RS218 and HM605 increased in numbers in the presence of amoeba suggesting the amoeba are sustaining bacterial growth (fig 3.8B). There were no significant differences ($P < 0.05$) in amoebal viability in the various bacterial co-cultures compared with amoebae alone (trypan blue analysis) (fig 3.8C). These findings show a direct correlation with grazing resistance and indicate that *A. polyphaga* are sustaining growth of AIEC HM605 whereas the numbers of MG1655 are significantly reduced.

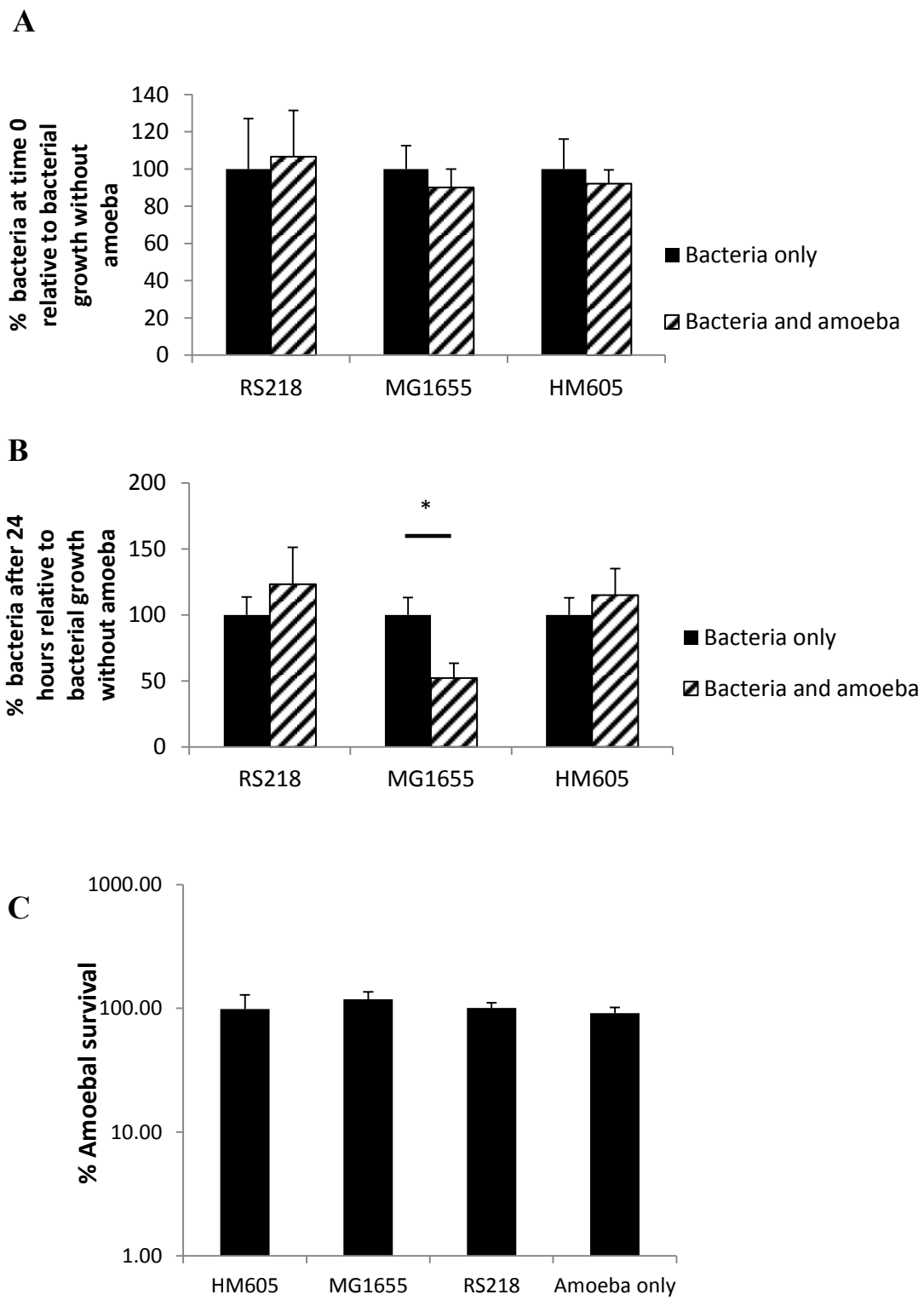


Figure 3.8: Co-interactions of bacteria and amoeba. A) Percentage bacteria at time 0 relative to growth without amoeba. No significant difference is observed as similar numbers are seen in all strains. B) Percentage bacteria after 24 hours relative to growth without amoeba. After 24 hours RS218 and HM605 show an increase in numbers in the presence of amoeba suggesting the amoeba are sustaining bacterial growth whereas MG1655 shows a decrease in numbers showing evidence of predation. Student's t-test ($P < 0.05$). C) Viability of *Acanthamoeba polyphaga* following 24 h of co-incubation with the test bacteria. Results illustrate percentage survival relative to control (absence of bacteria) amoebal counts. Results are from triplicate determinations.

3.3.6.1 Co-interactions of ECOR strains showing AIEC characteristics and amoeba

In addition to the previous experiment, co-culture/predation assays were conducted with the AIEC-like ECOR strains in order to further investigate if the amoeba *A. polyphaga* is sustaining the growth of these strains, similar to what is observed with HM605 (see Section 3.3.6). The co-culture experiments were set up as previously described (see Section 3.3.6 and Section 2.34). From the results, it can be that at T 0 no significant difference is observed as similar numbers are seen in all strains (fig 3.9 A). However, at 24 h post-infection all of the AIEC-like ECOR strains showed an increase in bacterial numbers in the presence of amoeba compared with controls in the absence of amoeba (see fig 3.9 B). This suggests that the amoeba is sustaining the growth of these strains similar to AIEC HM605. However, even ECOR-20 showed an increase despite the fact that this was reported as not resistant to grazing in Table 3.3. As expected MG1655 was significantly ($P < 0.05$) lower in numbers in the presence of the amoebae compared with controls in the absence of amoebae. This indicates the occurrence of predation as the amoebae are feeding on MG1655 (see fig 3.9 B). There were no significant differences ($P < 0.05$) in amoebal viability in the various bacterial co-cultures compared with amoebae alone (fig 3.9 C). These findings provide further evidence for the correlation of resistance to killing by amoeba with intramacrophage replication.

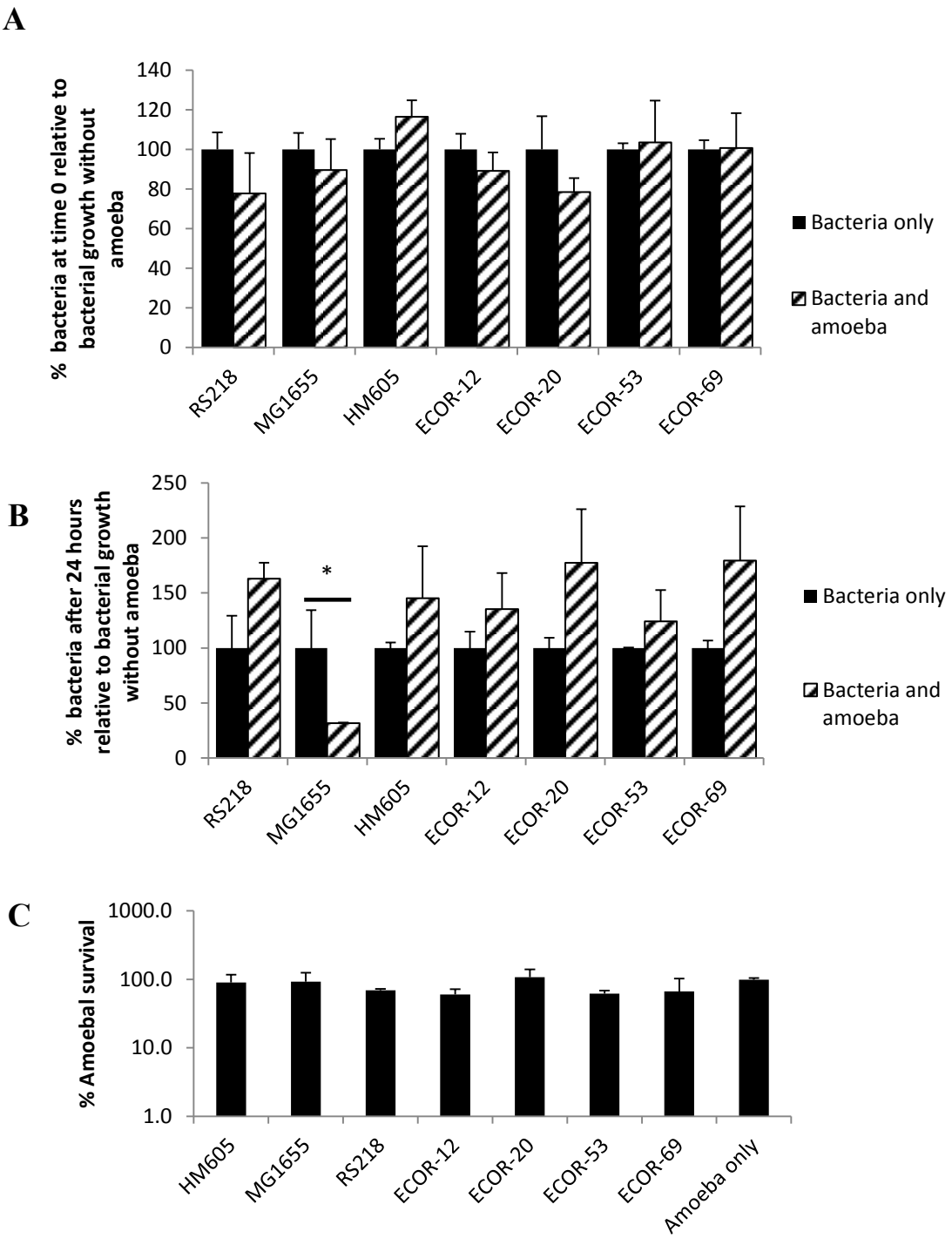


Figure 3.9: Co-interactions of ECOR strains showing AIEC characteristics and amoeba. A) Percentage bacteria at time 0 relative to growth without amoeba. No significant difference is observed as similar numbers are seen in all strains. B) Percentage bacteria after 24 hours relative to growth without amoeba. After 24 hours all strains show an increase in numbers in the presence of amoeba suggesting the amoeba are sustaining bacterial growth whereas MG1655 shows a decrease in numbers showing evidence of predation. Student's t-test ($P < 0.05$). C) Viability of *Acanthamoeba polyphaga* following 24 h of co-incubation with the test bacteria. Results illustrate percentage survival relative to control (absence of bacteria) amoebal counts. Results are from triplicate determinations.

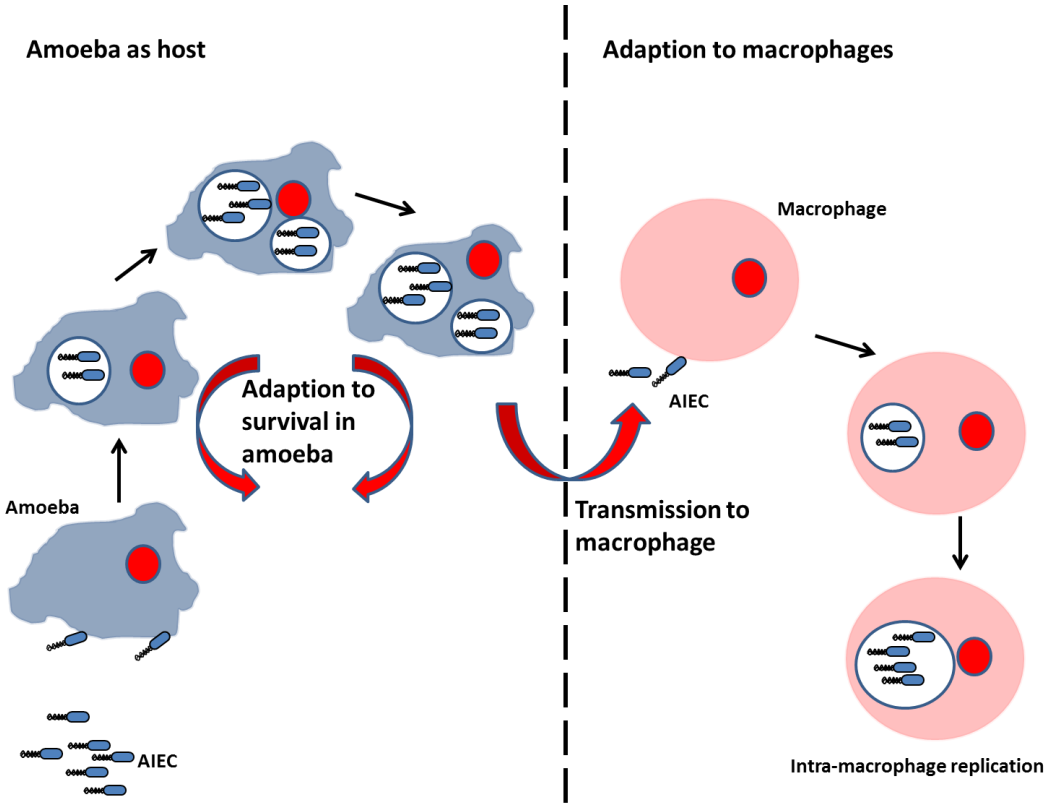


Figure 3.10: Proposed model of amoeba as evolutionary training ground for AIEC. *E. coli* encounters amoeba and becomes phagocytised. During the cycle of intra-amoebal replication, bacteria select virulence traits and adapt to survival with the amoeba. Moreover, amoebal vacuoles may represent an important reservoir of bacteria. *E. coli* adapted to survive in amoeba could then be transmitted and come into contact with macrophage. As a result they may be resistant to killing by macrophage and hence undergo intramacrophage replication

3.4 Conclusion

The AIEC pathotype has been repeatedly reported for its association with CD (Barnich *et al.*, 2007; Barnich & Darfeuille-Michaud, 2007a; Bringer *et al.*, 2006; Darfeuille-Michaud, 1998). Although studies have examined the relationship of AIEC with extraintestinal *E. coli* (ExPEC) (Martinez-Medina *et al.*, 2009a; Martinez-Medina *et al.*, 2009c) to date little is known on the genetic relationship between AIEC and other commensal and pathogenic strains of *E. coli*. This chapter focused on investigating the distribution of the AIEC phenotype within the *E. coli* genus. AIEC are characterised by their ability to colonise the intestinal mucosa by adhering to and invading intestinal epithelial cells and by their ability to survive and to replicate within macrophages (Bringer *et al.*, 2006; Bringer *et al.*, 2011; Darfeuille-Michaud, 1998). To determine the distribution of the AIEC phenotype, the ECOR collection and 12 other wildtype *E coli* strains were selected and examined for their ability to adhere to and invade intestinal epithelial cells, as well as their capacity to survive and replicate within macrophages. The advantage of using the ECOR collection is that it is a well-characterized strain set that is representative of the genetic diversity of the *E. coli* genus.

Initial findings concluded that the ability of *E. coli* to undergo intramacrophage replication is not a unique feature of AIEC; on the contrary it appears that the ability of *E. coli* to persist is widespread across the phylogenetic groups A, B1, B2 and D. On average the *E. coli* examined could undergo 2-fold replication inside the macrophage. Further characterisation of the intramacrophage replication phenotype identified that a number of strains had the ability to reproducibly replicate $\geq$1 standard deviation greater than the average to levels similar to a known AIEC strain. These 5 strains could therefore be potentially characterised as AIEC due to their ability to replicate at levels similar to other AIEC strains such as HM605. Each of these strains was distributed across the phylogenetic groups. These findings support the previous observation that AIEC is not a clonal group of *E. coli* (Martinez-Medina *et al.*, 2009a; Sepehri *et al.*, 2009). All AIEC strains isolate to date are reported to be diverse and belong to distinct serotypes (Martinez-Medina *et al.*, 2011). Furthermore, even though they primarily fall into the B2 group, AIEC strains belonging to groups A, B1, and D have also been isolated (Martinez-Medina *et al.*, 2011). As the secondary screen removed a number of false positives it is important

to note that the primary screen possibly gave some false negatives. Therefore, 5 strains are likely to be an underestimation of the real number. However, this does not detract from the findings presented here that the ability to replicate is widespread in the *E. coli* genus. Of these strains, 4 showed an ability to adhere and invade C2Bbe1 epithelial cells. In total, 4 out of 84 (4.96%) strains from our collection were found to share the phenotypic characteristics that describe the AIEC pathovar. These results suggest that approximately 5% of the *E. coli* genus show AIEC phenotype. This is significant as *E .coli* possessing AIEC characteristics could constitute the normal flora of an individual and as AIEC strains have been found in healthy controls it suggests that they behave as opportunistic pathogens in CD patients. Furthermore, recent studies have identified AIEC in the intestine of several animal species suggesting a putative zoonotic risk and provide support for the absence of host specificity of this pathotype (Martinez-Medina *et al.*, 2011; Simpson *et al.*, 2006). Although some genes have been implicated in the pathogenesis of AIEC (Barnich *et al.*, 2003; Barnich *et al.*, 2004; Bringer *et al.*, 2005), these genes are found in non-pathogenic *E. coli* K-12 strains therefore pathoadaptive mutations or difference in gene expression could contribute to the characteristics of AIEC. This hypothesis is supported by previous reports showing that considerable intraspecies genomic size variation of *E. coli* is found in different strains indicating the addition or deletion of genes for an adaptive phenotype (Sepehri *et al.*, 2009).

Phagosome acidification is an important component of the microbicidal response of eukaryotic cells (Steele-Mortimer *et al.*, 2000). Intracellular pathogens must either block phagosome acidification or be able to survive at low pH. In *Salmonella enterica* serovar Typhimurium, vacuolar acidification may only play a role in intracellular survival of certain salmonellae only under certain conditions and in specific cell types as acidification was essential for *Salmonella* intracellular survival in J774.A1 cultured macrophages but not essential in other macrophage cell lines (Steele-Mortimer *et al.*, 2000). Therefore, in order to investigate if acidification of the phagosome is required for intracellular replication of the four ECOR strains with AIEC characteristics, the effect of the vacuolar pH-neutralizing reagent ammonium chloride (NH_4Cl) on the replication ability within J774 macrophages was examined. It was concluded that some strains intracellular replication ability is pH dependent as they require acidification of the phagosome such as HM605 and ECOR-69, whereas

strains such as ECOR-20 and ECOR-53 ability to replicate appear to be pH independent (See Fig 3.4). ECOR-12 should little to no change. Acid vacuolar pH has been reported to play a key role in the AIEC LF82 replication within macrophages (Bringer *et al.*, 2006). However, as only AIEC LF82 was examined in previous studies it was not clear if acidification of the phagosome was a phenotype of other AIEC. The findings presented here would suggest that AIEC is phenotypically diverse as 3 different requirements for phagosome acidification were identified. Furthermore, as all strains can undergo intramacrophage replication these results seem to suggest that phagosome acidification and tolerance to low pH are not essential for the intracellular replication of all *E. coli*.

Further characterisation of the ECOR strains sharing AIEC characteristics focused on the detection of cytokines TNF-α and IL1-β in the supernatant of infected J774.A1 macrophages. Previous studies suggest that AIEC LF82-infected macrophage secrete large amounts of tumour necrosis factor-alpha (TNF-α), which in turn increases the intramacrophagic replication of LF82 (Bringer *et al.*, 2011; Glasser *et al.*, 2001). However, as previously stated no *E. coli* K-12 control was used in any of these studies to obtain a level of cytokine production of a commensal, non-intramacrophage replicating *E. coli* relative to LF82. Values were only compared to LPS-stimulated macrophage or non-replicating mutants of LF82. Therefore, in these studies it is not clear if the TNF-α level induced by LF82 is a characteristic of AIEC or all *E. coli* as TNF-α is one of the highest induced cytokines in macrophage in response to bacterial peptidoglycan (PGN) (Xu *et al.*, 2001). The purpose of conducting cytokine analysis on the ECOR strains was to attempt to elucidate if high levels of TNF-α production are induced by these strains similar to AIEC LF82 or if IL1-β levels are induced in these strains. Cytokine analysis revealed that at 24 hours post-infection no significant difference was observed between the *E. coli* K-12 control and AIEC HM605 or any ECOR strain. All strains induced TNF-α production to similar levels. As stated, it has been reported that TNF-α is induced by macrophage in response to bacterial PGN and PGN-induced expression of TNF-α is directly mediated through the transcription factor NF_KB and indirectly through the transcription factor Elk-1 (Xu *et al.*, 2001). This data would suggest that the increase in TNF-α induction by all *E. coli* examined could just be in response to bacterial

PGN and no significant amount of TNF-α is produced by infected J774.A1 macrophage. However, the results from the cytokine analysis revealed that 24 hours post-infection, AIEC HM605 and the ECOR infected J774.A1 macrophage induced a significantly higher release of IL1-β in comparison to the commensal *E. coli* K-12 control. Secretion of IL-1β, a potent pyrogen that elicits a strong pro-inflammatory response, is tightly controlled by a diverse class of cytosolic complexes known as inflammasomes (Shimada *et al.*, 2011). IL1-β is first synthesized as biologically inactive pro-IL-1beta, then processed into mature, biologically active IL1-β by caspase-1, and subsequently released (Eder, 2009). This pro-inflammatory reaction is a key element of pyroptosis, cell death seen in response to infection by the pathogens *Salmonella enterica*, *Shigella flexneri*, and *L. pneumophila (Whitfield et al., 2009).* Until recently, very little was known of the ability of bacteria to interfere with or to utilize extracellular cytokines secreted by the host cells or intracellular cytokines within phagocytic cells (Meduri *et al.*, 2001). Studies have reported enhancement of bacterial growth in the presence of IL-1β for *E. coli (Porat et al., 1991).* Similarly, the intracellular bacterial pathogen *Chlamydia trachomatis* triggers the secretion of IL-1β (Kavathas *et al.*, 2012). The findings presented here suggest that intramacrophage replicating *E. coli* induce significantly higher amounts of IL1-β, not TNF-α in comparison to a non-replicating *E. coli* K-12 control. Therefore the production of IL1-β appears to be specific for AIEC.

It has been previously suggest that grazing resistance to protozoa was an evolutionary precursor of bacterial pathogenicity (King *et al.*, 1988). For example, it was recently shown that the Shiga toxin genes carried by some intestinal pathogenic *E. coli* strains increase bacterial survival in presence of the protozoa *Tetrahymena pyriformis* indicating that such interactions might have driven the evolution of this virulence factor (Adiba *et al.*, 2010; Steinberg & Levin, 2007). As stated, the coincidental evolution hypothesis suggests that virulence traits in bacteria might result from selective pressure exerted by protozoan predators such as amoeba (Adiba *et al.*, 2010). To help examine the hypothesis that AIEC could have evolved the ability to replicate in macrophage via their interaction with amoeba, AIEC HM605 and the intramacrophage replicating ECOR strains were examined for their ability to resist killing by amoeba. The results from the grazing resistance assay showed that the intramacrophage replicating AIEC strain HM605 is resistant to killing by the

amoeba *A. polyphaga*. In contrast the non-replicating commensal *E. coli* K-12 control was readily killed by the amoeba. The co-culture results show a direct correlation with grazing resistance and indicate that *A. polyphaga* are sustaining growth of AIEC HM605 whereas the numbers of MG1655 are significantly reduced. Similarly, the grazing resistance results for the ECOR strains revealed that approximately 75% (3/4) of the intramacrophage replicating ECOR strains show grazing resistance phenotype similar to HM605. In contrast, only 12.5% (1/8) of non-intramacrophage replicating ECOR strains showed grazing resistance phenotype. The selection of virulence traits may explain the observed adaptation to macrophages of most microbes able to survive and grow intracellularly in free-living amoebae (Greub & Raoult, 2004). For example, there are large similarities between the way in which *Legionella* sp, adapted to both macrophage and amoeba (Greub & Raoult, 2004). The absence of phagosome-lysosome fusion is characteristic of the survival of *Legionella* in both macrophage and amoeba (Bozue & Johnson, 1996; Greub & Raoult, 2004; Horwitz, 1983). Moreover, the *rtxA* gene, which has been implicated the formation of pores in both human and murine monocytes and in intracellular replication in human monocytes (Greub & Raoult, 2004) was shown to be implicated in both adherence to and entry into *A. castellanii* (Cirillo *et al.*, 2002). Similarly, *Mycobacterium avium* mutants lacking pathogenicity islands exhibited highly similar defects in invading *Acanthamoeba castellanii* and human macrophages (Salah *et al*., 2009).

In conclusion the results presented here indicate a correlation between survival in macrophage and resistance to killing by amoeba. Our observations support the hypothesis that amoeba may act as an evolutionary 'training ground' for AIEC as significantly more intramacrophage replicating strains showed grazing resistance phenotype. Selective pressures placed by amoebae on amoeba-resistant AIEC may have been involved in the evolution of virulence in this group with the hypothesis that adaptation to life within human macrophages may be possible following exposure to environmental predators such as free-living amoebae. The results presented here support the hypothesis that the variety of survival strategies used by intracellular bacteria for resisting macrophages may reflect the long co-evolution process of bacteria and amoebae (Greub & Raoult, 2004). The possible involvement of amoeba in the evolution of AIEC phenotype is also important for ecological and

epidemiological reasons. Amoeba and bacteria are in a closed relationship in their different habitats and the capacity to resist grazing by protozoa may manifest itself in a human host as increasing virulence (Adiba *et al.*, 2010). As protozoa are widely distributed in water, soils and effluents, they may constitute an important environmental reservoir for transmission and acquisition of *E. coli* possessing AIEC characteristics and other pathogens from the environment.

Chapter 4.0: Role of the Cpx A/R two component pathway in the pathogenesis of adherent invasive *Escherichia coli* strain HM605

4.1 Introduction

For any bacteria, the key to successful adaptation to environmental variation is to recognise signals and rapidly adapt gene expression profiles to new situations (Szurmant *et al.*, 2007). Two component signal transduction systems are an important means by which bacteria sense and respond to a wide variety of external signals, stressors and other changes in the environment (Capra & Laub, 2012; Laub & Goulian, 2007). Two component systems (TCS) are typically composed of a sensor histidine kinase (HK) which, in response to an input stimuli autophosphorylates and transfer the phosphoryl group to a DNA-binding response protein known as the response regulator (RR) that can than effect appropriate changes in gene expression and cellular physiology (see fig 4.1) (Capra & Laub, 2012; Laub & Goulian, 2007; Szurmant *et al.*, 2007). Both the HK and RR have intrinsic modularity that separates signal input, phosphotransfer, and output response (Capra & Laub, 2012). Environmental stimuli are detected either directly or indirectly by the N-terminal sensing domain of the HK. These diverse sensing domains share little primary sequence similarity, suggesting a role in specific ligand/stimulus interactions (Stock *et al.*, 2000). The majority of HKs are transmembrane spanning proteins, which in principle allow for the detection of extracellular signals and a C-terminal transmitter kinase domain catalyses the autophosphorylation at a conserved histidine residue in an ATP dependent manner (Mizuno, 1997). Most RRs consist of two domains: a conserved N-terminal regulatory domain and a variable C-terminal effector domain (Stock *et al.*, 2000). The conserved N-terminal receiver domain of RRs serves as a phospho-accepting domain, in which an invariant aspartate residue is located. The receiver domain catalyses transfer of a phosphoryl group from the phospho-histidine of the HK to this aspartate residue (see fig 4.1) (Gao *et al.*, 2008; Mizuno, 1997).

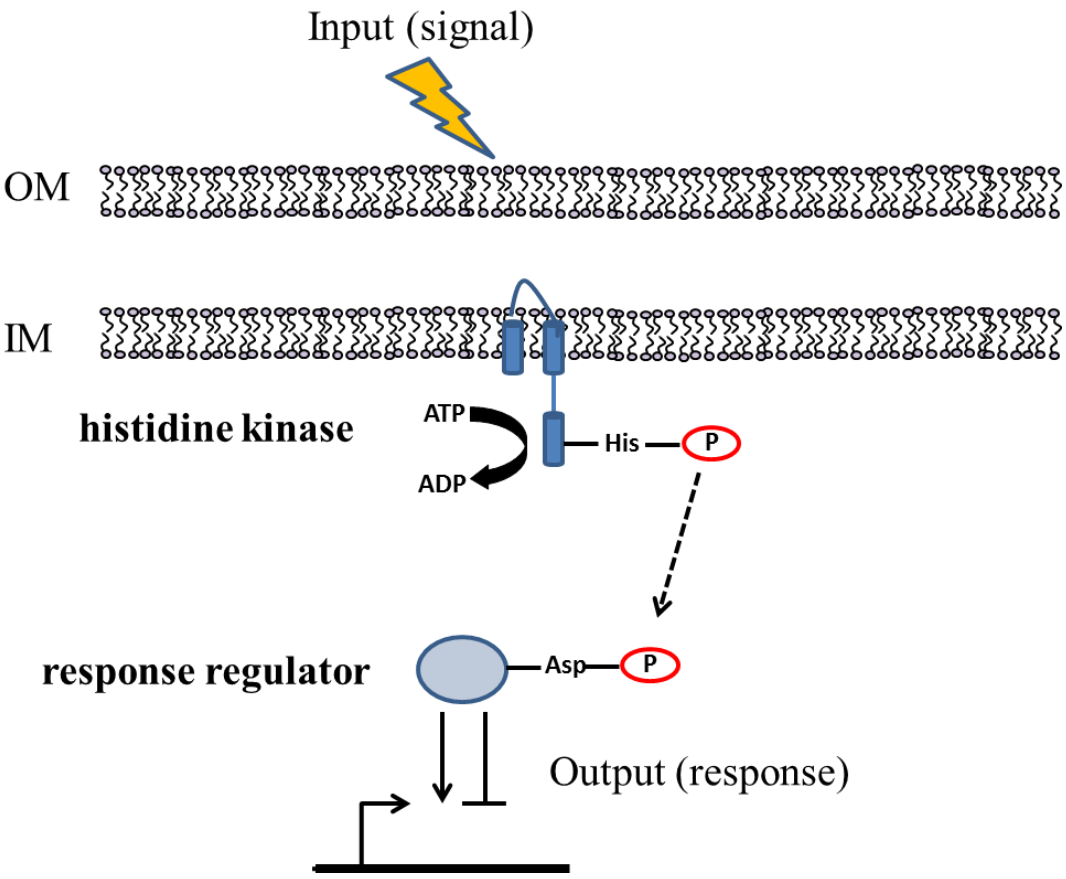


Figure 4.1: Two component signal transduction.
Receipt of an external input signal stimulates autophosphorylation of a histidine kinase (HK). The phosphoryl group is subsequently passed to a cognate response regulator (RR), which can trigger changes in gene expression or other cellular physiological processes (output response).

The phosphorylated RR functions as the ultimate control element that modulates the activity of its output domain and elicits the particular response. The majority of effector domains have DNA-binding activity and function to activate and/or repress transcription of specific genes. However, the specific DNA sequences that are recognized and the specific mechanism of transcriptional regulation differ for each RR (Stock *et al.*, 2000). In addition, a small number of two component pathways contain a histidine-containing phosphotransfer domain (HPt) composed of approximately 120 amino acids and a short consensus motif in which an invariant histidine residue is located (Mizuno, 1997). This His residue is capable of participating in phosphoryl transfer reactions (Mizuno, 1997). The HPt domains do not exhibit kinase or phosphatase activity and are ideally suited to serve as specific cross-communication modules between different proteins (Stock *et al.*, 2000; Tsuzuki *et al.*, 1995). Although TCS are present in organisms from all domains of life, His-Asp phosphotransfer systems account for the majority of signalling pathways in the Bacteria (Stock *et al.*, 2000). To date there are approximately 30 HKs (5 of which are hybrid kinases) and 34 RRs in *E. coli* MG1655 and it is reported that both lineage-specific expansion and horizontal gene transfer have contributed to the evolution of the diversity of TCS that allow bacteria to adapt to complex environments (Alm *et al.*, 2006; Gao *et al.*, 2008; Mizuno, 1997; Zhao *et al.*, 2009).

4.1.1 Two-component pathways and virulence

During the course of infection, bacteria must co-ordinately regulate gene expression in response to environmental stimuli and consequently gene clusters contributing to processes such as cell growth, biofilm formation, drug resistance, virulence factor expression and pathogenicity are often controlled by TCS (Crepin *et al.*, 2011; Gotoh *et al.*, 2010; Kawada-Matsuo *et al.*, 2011; Watanabe *et al.*, 2008). For example, a number of studies have highlighted the importance of the phosphate (Pho) regulon in bacterial pathogenesis (Crepin *et al.*, 2011; Lamarche *et al.*, 2008). The Pho regulon plays a key role in phosphate homeostasis and it is controlled by the PhoR/B TCS (Lamarche *et al.*, 2008). The phosphate-specific transport (Pst) system belongs to the Pho regulon and several studies have linked Pst with virulence

(Lamarche *et al.*, 2008). Deletion of the *pst* genes in an ExPEC strain belonging to the avian pathogenic *E. coli* (APEC) group led to constitutive activation of the Pho regulon and was shown to reduce virulence in a chicken infection model (Lamarche *et al.*, 2005). Furthermore, inactivation of *phoB* in *Vibrio cholerae* showed a decreased capacity for colonization of adult rabbit ligated ileal loops in competitive colonization assays with the wild-type strain indicating a role for PhoR/PhoB as a modulator of virulence attributes (Crepin *et al.*, 2011).

In addition the TCS PhoP/Q has also been shown to play a role in the virulence and polymyxin resistance of a number of Gram negative species such as *E. coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium (Gellatly et al., 2012; Golubeva & Slauch, 2006; Moon & Gottesman, 2009). PhoQ is a sensor kinase that phosphorylates and activates the transcription factor PhoP (Lippa & Goulian, 2009). The PhoP/Q TCS acts as a transcriptional regulator that responds to Mg^{2+} starvation and PhoP is considered a virulence regulator despite being conserved in both pathogenic and non-pathogenic *Enterobacteriaceae* (Monsieurs *et al.*, 2005). In *P. aeruginosa* a phoQ mutant co-cultured on epithelial cells produced less secreted protease and lipase and demonstrated reduced cytotoxicity relative to wild-type (Gellatly *et al.*, 2012). In addition, it was established that PhoP/Q is directly involved in the regulation and expression of the SPI-2 pathogenicity island of *Salmonella enterica* demonstrating that the Mg^{2+}-modulated system plays an important regulatory role in *Salmonella* during infection (Aguirre *et al.*, 2006; Bijlsma & Groisman, 2005).

The EnvZ/OmpR TCS was found to modulate virulence in *S. flexneri* according to environmental conditions (Bernardini *et al.*, 1990; Bernardini *et al.*, 1993). In *S. flexneri*, the *ompB* locus (containing the *ompR* and *envZ* genes) was found to regulate the expression of the *vir* genes, which are responsible for invasion of epithelial cells. The expression of the *vir* genes was up-regulated under conditions of high osmolarity, similar to that encountered in tissues, both extra- and intracellularly (Bernardini *et al.*, 1990; Bernardini *et al.*, 1993). Similarly, at high osmolarity, increased expression of OmpC in AIEC LF82 bacteria and activation of the σ^E regulatory pathway are also observed (Rolhion *et al.*, 2007). This activation modulates AIEC virulence factors, flagella and/or type 1 fimbriae

encoding gene expression involved in the interaction of LF82 with host epithelial cells (Rolhion *et al.*, 2007). Other TCS such as BarA/UvrY have also been implicated in the pathogenesis of Gram negative bacteria such as avian pathogenic *E. coli* (APEC) (Herren *et al.*, 2006). Mutations in *barA* and *uvrY* significantly reduces APEC survival within chicken macrophages as well as increasing susceptibility to oxidative stress and reducing expression of exopolysaccharide and pili (Herren *et al.*, 2006). These studies highlight the importance of two-component signal transduction systems as modulators of virulence in pathogenic bacteria.

Finally recent evidence indicates that the CpxA/R TCS is frequently involved in regulating structures required for virulence in pathogenic Gram-negative bacteria (Raivio, 2005). Moreover, some of these effects may be resulting from Cpx regulation of major virulence regulatory proteins in these organisms (Raivio, 2005). The CpxA/R TCS is an envelope stress response system that mediates a response to envelope protein misfolding (Price & Raivio, 2009). CpxA/R TCS comprises the inner membrane sensor CpxA (HK) and the cytoplasmic cognate response regulator CpxR (RR) (Dorel *et al.*, 2006; Raivio, 2005). The CpxA/R pathway has been shown to be important for the assembly and expression of the P pilus of UPEC, which mediates UPEC adherence to kidney cells (Hung *et al.*, 2001). Expression of misfolded PapG and PapE pilin subunits up-regulates the CpxA/R TCS. Upon sensing subunit misfolding of pilus in the periplasm, the CpxA/R TCS increases the expression of degP and dsbA, encoding proteins that are required for efficient pilus assembly (Jones *et al.*, 1997; Raivio, 2005). DegP is a protease that degrades misfolded pilins, and DsbA is an enzyme that catalyses the disulfide bond formation required for the assembly of P pili (Jones *et al.*, 1997). Furthermore, *cpxR* null mutations and P pilus overexpression plasmids induce P pili that are dramatically shorter than those of the wildtype strain (Hung *et al.*, 2001; Raivio, 2005). In addition, the CpxA/R TCS has also been shown to be involved in the expression of the type IV bundle-forming pilus (BFP) of EPEC (Leuko & Raivio, 2012; Vogt *et al.*, 2010). EPEC strains lacking CpxR exhibit reduced BFP expression and diminished auto-aggregation and adherence to eukaryotic cells, both of which are BFP-mediated (Raivio, 2005; Stock *et al.*, 2000; Vogt *et al.*, 2010). Activation of the Cpx A/R TCS upon pilus expression appears to be an important mechanism in

ensuring efficient assembly of the P pilus, likely by providing necessary folding and degrading factors (Nevesinjac & Raivio, 2005). Moreover, a recent study has shown that mutations that activate the CpxA/R TCS have a dramatic effect on the ability of EPEC to establish a lethal infection in wax moth larvae *Galleria mellonella* (Leuko & Raivio, 2012). Activation of the Cpx A/R TCS in EPEC decreases transcription of several operons on the locus of enterocyte effacement (LEE) pathogenicity island inhibiting components of the type 3 secretion apparatus, the bacterial adhesin intimin, and the translocated bacterial receptor Tir, required for the adherence and invasion of epithelial cells (Macritchie *et al.*, 2008).

4.2 Objectives

TCS can alter gene expression in response to environmental changes and are well characterised as having an important role in regulating many bacterial characteristics such as virulence (Gotoh *et al.*, 2010). Therefore, the objective of this Chapter was to determine the potential role of TCS in the pathogenesis of AIEC strain HM605.

4.3 Results

4.3.1 Response regulator mutants of AIEC HM605

In order to facilitate a role for two component pathways in the pathogenesis of HM605, deletion mutants in genes encoding predicted RR were constructed using P1 bacteriophage transduction (see Section 2.22). Donor strains containing the desired mutant allele were obtained from the KEIO collection (Baba *et al.*, 2006). The KEIO collection comprises a set of single gene in-frame deletion mutants of most non-essential genes (3,985 in total) in *E. coli* BW3115 and therefore provides a useful resource for the transfer of mutant alleles to recipient strains via P1 transduction. In the KEIO library individual genes have been replaced with a kanamycin resistance cassette flanked by FRT (FLP recombinase target) sequences. The FRT sequences enable excision of the resistance gene by site-specific recombination using the FLP recombinase on plasmid pFLP3 (Baba et al., 2006). In total, 11 mutants were constructed (see table 4.1). TCS are not essential in *E. coli* and one possible reason for the inability to construct all RR mutants could be an unexpected loss of synteny with the genome of *E. coli* BW3115. In order for successful transduction to occur, the location of the desired gene must be in the same location in both donor and

recipient strains. Therefore, loss of synteny with the genome of *E. coli* BW3115 could affect the transfer of genomic material to HM605.

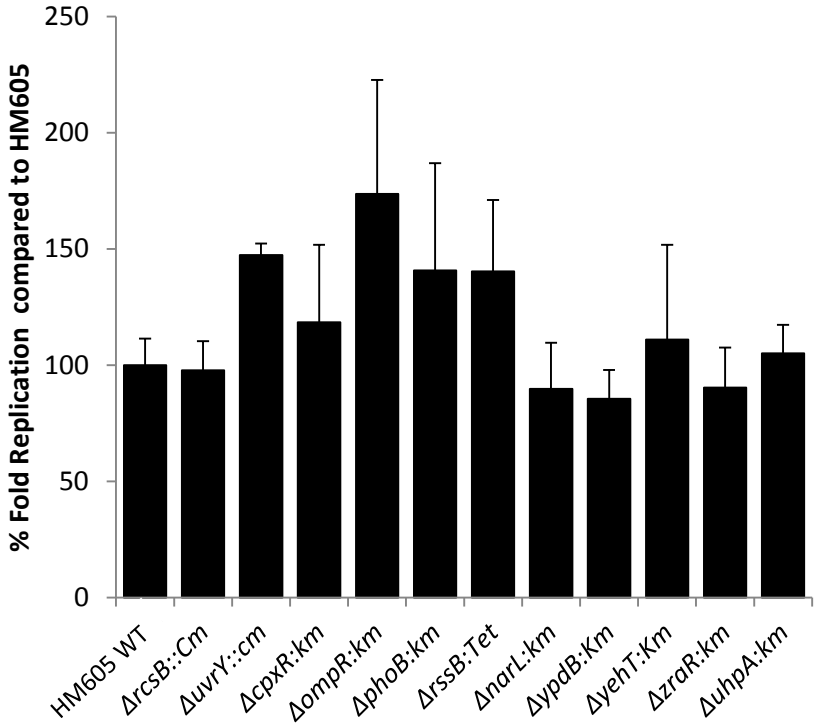
Table 4.1: Response regulators mutants of AIEC strain HM605

Response regulator	Function
ΔcpxR::Km	Envelope stress response
ΔompR::Km	Adaption to changes in osmolarity.
ΔphoB::Km	Response to environmental phosphate levels.
ΔzraR::Km	Senses high periplasmic metal levels (Zn).
ΔuhpA::Km	Unknown.
ΔnarL::Km	Nitrate/nitrite response regulator. Pleiotrophic regulation of anaerobic respiration.
ΔypdB::Km	Unknown.
ΔyehT::Km	Drug resistance.
ΔrcsB::Cm	Colanic acid capsule synthesis.
ΔrssB::Cm	Regulates degradation of Sigma S.
ΔuvrY::Cm	DNA repair and carbon metabolism.

4.3.2 All RR mutants tested were non-essential for intramacrophage replication of AIEC HM605.

To date, little is known how AIEC survive phagocytosis by the macrophage and, therefore, the first objective of this study was to examine the 11 response regulator mutants for their ability to replicate inside J774.A1 macrophage using the gentamicin protection assay (see section 2.29). From the data presented in Figure 4.2A it was observed that there was no significant difference between the fold replication of the response regulator mutants compared to wildtype AIEC HM605. Therefore, these findings indicate that none of the RR examined play a role in the survival of AIEC HM605 in J774.A1 macrophages. However, the *cpxR* mutant showed a statistically significant lower percentage of internalised bacteria 1h post-gentamicin compared to wildtype HM605 (see fig 4.2 B). Interestingly, this result implies that although the deletion of *cpxR* does not affect the ability of AIEC HM605 to replicate inside macrophage, this mutation does appear to have an effect on the ability of the macrophage to internalise the mutant bacteria.

A



B

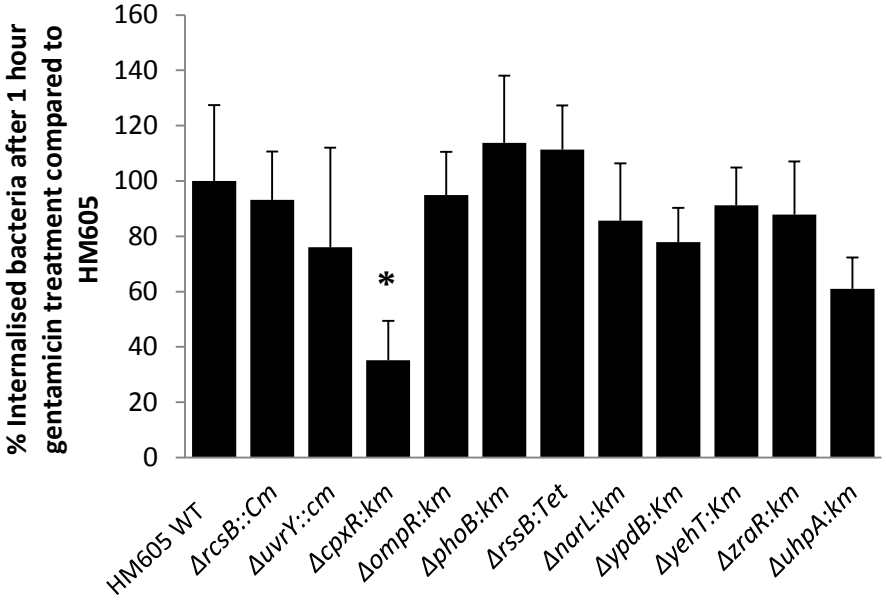
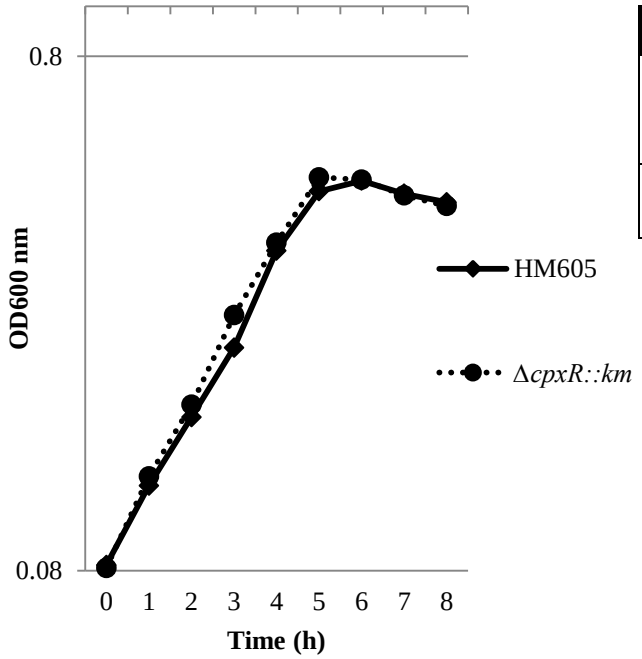


Figure 4.2: 11 response regulator mutants HM605 are examined for their ability to replicate in J774.A1 macrophages.

A) Bacterial survival and replication after 6 h of gentamicin treatment. All response regulator mutants could undergo intramacrophage replication. Results are expressed as the number of intracellular bacteria at 6 h relative to that obtained at 1 h after gentamicin exposure, taken as 100%. **B) Internalization of wild-type and mutant bacteria.** HM605 carrying a mutation in the *cpxR* response regulator is reduced in its uptake by J774.A1 macrophages but is not reduced in its ability to replicate (see (A)). Results are expressed as the number of internalised bacteria after 1 h of gentamicin treatment relative to that obtained for the wild-type, taken as 100%. For analysis of the significance of differences in uptake and survival within J774-A1 macrophages, Student's t-test was used for comparison of two groups of data (∗ P-value ≤ 0.05).

Although this defect in internalisation is probably due to surface changes arising from the inactivation of the CpxA/R TCS, it is formally possible that the mutant bacteria may be stressed during internalization and therefore harder to recover from the macrophage. However, we have shown that the growth rate of the *cpxR* mutant is indistinguishable from the wild-type in both DMEM and the macrophage suggesting that the *cpxR* mutant is not stressed in the macrophage (see fig. 4.3).

A



B

Strain	Growth rate (h^{-1})
HM605	0.38
ΔcpxR:km	0.41

Figure 4.3: A) Growth of the wild-type strain HM605 and of HM605Δ*cpxR*::Km in DMEM medium supplemented with 10% FBS. Deletion of the *cpxR* gene does not affect the growth in HM605Δ*cpxR*::Km. **B) Growth rate of HM605 and of HM605Δ*cpxR*::Km inside J774.A1 macrophage.** Both wildtype and *cpxR* mutant show similar growth rates inside macrophage indicating that the *cpxR* mutant is not affected in intramacrophage replication.

4.3.3 Sensitivity of *cpxR* mutant to external copper

In *E. coli* K-12 (MC4100) it has been shown that the CpxA/R TCS is involved in the response to copper stress. In particular it has been previously shown that a *cpxR* mutant is more sensitive to copper stress than a wild type strain (Yamamoto & Ishihama, 2005; Yamamoto & Ishihama, 2006). To determine whether this phenotype was solely specific for the K-12 strain, overnight cultures of HM605 and HM605 *ΔcpxR::*Km were diluted to OD_{600} =1.0 in fresh LB. The diluted culture was then serial diluted in 1 X PBS and plated onto LB agar containing 0.8 mM copper sulphate ($CuSO_4$). HM605 *ΔcpxR::*Km was significantly more sensitive to external copper than wildtype suggesting that the CpxA/R TCS is involved in mediating resistance to copper stress in HM605 (see fig 4.4).

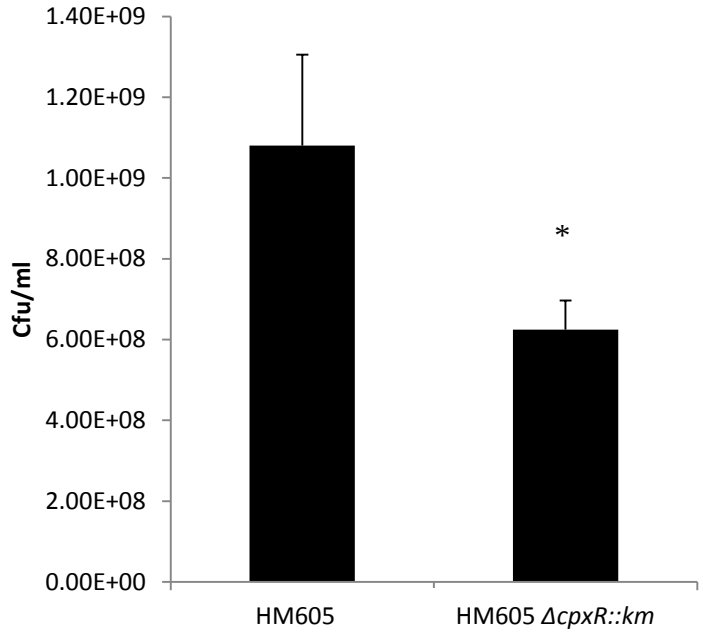


Figure 4.4: The *cpxR* mutant is more sensitive to external copper Overnight cultures of HM605 and HM605 *ΔcpxR::*Km were diluted to OD_{600} =1.0 in fresh LB. The diluted culture was then serial diluted in 1 X PBS and plated onto LB agar containing 0.8 mM $CuSO_4$. The graph represents the mean results of 3 cultures per strain replicated in triplicate. For analysis of the significance of differences in numbers, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

The expression of two genes, *yebE* and *ycfS*, has also been shown to be induced by the addition of copper, in a CpxA/R dependent manner (Yamamoto & Ishihama, 2005; Yamamoto & Ishihama, 2006). YcfS is predicted to be localized to the periplasm and contains a potential inner-membrane-anchoring sequence together with a LysM motif that may mediate an interaction with peptidoglycan (Kaserer *et al.*, 2008; Price & Raivio, 2009). YebE is predicted to be an inner membrane protein (Price & Raivio, 2009). The expression of *ycfS* and *yebE* was measured using promoter-GFP fusions taken from an *E. coli* promoter collection made by the Weizmann Institute of Science (Zaslaver *et al.*, 2006). The *E. coli* promoter collection is a library of transcriptional fusions of *gfp* to each of approximately 2,000 different promoters in *E. coli* K12. Each promoter fusion is expressed from a low-copy plasmid (pUA66) (Zaslaver *et al.*, 2006). As the pUA66 plasmid carries a kanamycin cassette, the kanamycin marker in HM605 *ΔcpxR::*Km was removed. The kanamycin marker is flanked two FRT sites which allow the antibiotic marker to be eliminated with the use of a FLP expression plasmid. The kanamycin resistance gene can be eliminated by using the helper plasmid pFLP3 expressing the FLP recombinase, which acts on the directly repeated FRT (FLP recognition target) sites flanking the kanamycin resistance gene (see Chapter 2 Fig 2.2) (Baba *et al.*, 2006). To analyse activation of the CpxA/R pathway, plasmids carrying the promoters of *yebE* and *ycfS* fused to GFP were transformed into HM605 and HM605 *ΔcpxR*. Cultures were incubated at 37°C in 96-well microtitre plates until OD_{600}=0.2 and, where appropriate, 0.5mM $CuSO_4$ was added to the wells. The cultures were incubated at 37°C and OD_{600} and GFP fluorescence was measured every 15 min over a period of 24 h using a MWG Sirius HT plate reader. As can be seen in Fig 4.5, there was a significant increase in the expression of both *ycfS* and *yebE* in HM605, after the addition of $CuSO_4$. In contrast, there was little or no increase in expression observed in the *cpxR* mutant. Therefore, both copper resistance and the copper-dependent expression of certain genes are dependent on the CpxA/R TCS in HM605.

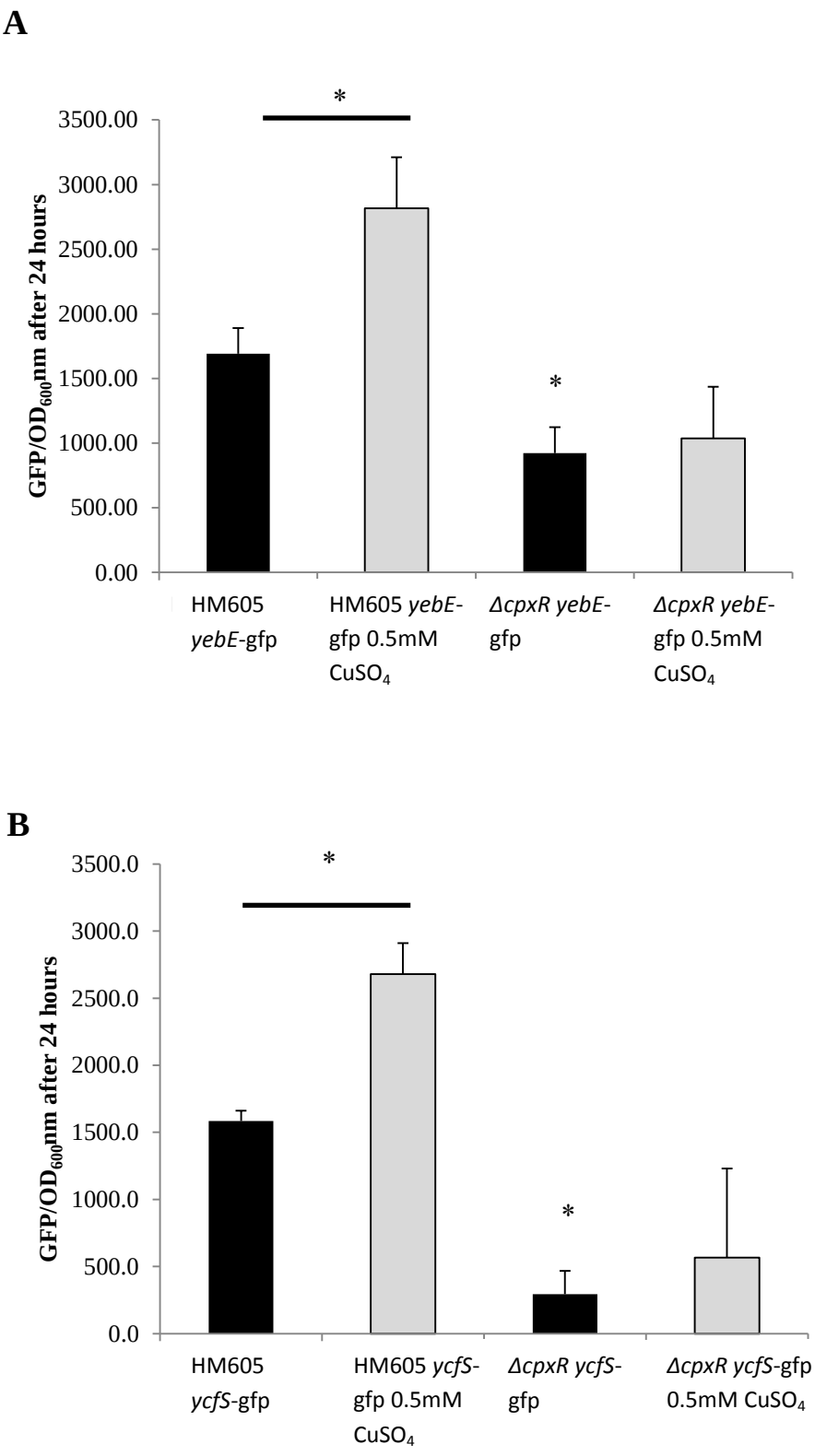


Figure 4.5: GFP/OD_{600}(nm) showing the levels of GFP expression using A) *yebE*-gfp reporter gene and B) *ycfS*-gfp reporter gene after 24 hours. In the wildtype, activation of the wildtype cpxA/R TCS using copper sulphate shows a significant increase in GFP expression as the pathway is activated whereas in the *cpxR* mutant little to no increase in GFP expression is observed as the pathway has been disrupted. Each strain was represented by 3 individual cultures). Black lines refer to addition of $CuSO_4$. For analysis of the significance of differences in expression, Student's t-test was used for comparison of two groups of data (∗ P-value ≤ 0.05).

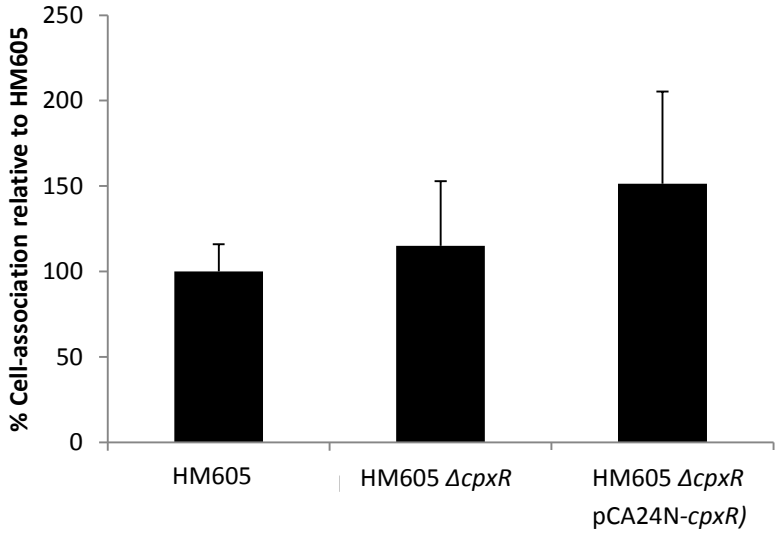
4.3.4 Adhesion and invasion of C2Bbe1 epithelial cells

As previously mentioned, the CpxA/R TCS is an envelope stress response system that mediates a cellular response to envelope protein misfolding (Price & Raivio, 2009). As such the main function of this pathway is to respond to damage to the cell envelope via the production of proteases and chaperones (Dorel *et al.*, 2006; Price & Raivio, 2009). As already discussed it has been well documented that the CpxA/R TCS plays an important role in the virulence of pathogenic Gram negative bacteria (De Wulf *et al.*, 1999; Raivio, 2005). In particular the CpxA/R TCS has been linked to adhesion of EPEC to epithelial cells through the expression of the type IV bundle-forming pilus (BFP) (Leuko & Raivio, 2012; Vogt *et al.*, 2010). As the CpxA/R TCS appears to play a role in the initial interaction between HM605 and the J774.A1 macrophage, the next stage was to examine HM605Δ*cpxR* for its ability to adhere to, and invade, C2Bbe1 epithelial cells.

4.3.4.1 HM605 *ΔcpxR* is reduced in its ability to invade C2Bbe1 epithelial cells.

To test for an effect on adherence and invasion, bacteria were incubated with C2Bbe1 epithelial cells at an M.O.I of 10 at 37C for 3 h. Following infection, monolayers were washed twice in sterile 1X PBS, lysed with 1% (v/v) Triton X-100 and plated onto LB plates to determine the total number of cell-associated bacteria. To specifically determine the number of intracellular bacteria, cell culture medium containing 100μg/ml gentamicin was added to the wells for 1 h in order to kill any extracellular bacteria and the bacteria were quantified as described above. No significant difference in the numbers of cell-associated bacteria (adherent plus intracellular) was observed between the wild-type and the *cpxR* mutant since the mean numbers of cfu per well were $1.86 \times 10^6 \pm 3.15\ 10^5$ and $2.26 \times 10^6 \pm 7.46\ 10^5$, respectively (see fig 4.5A). However, the ability of the Δ*cpxR* mutant to invade C2Bbe1 epithelial cells was significantly ($P \leq 0.05$) impaired (see fig 4.5B). The percentage of invaded bacteria for the *cpxR* mutant relative to the wild type was 37.13% ± 13.5%. Importantly, complementation of the Δ*cpxR* mutant with a cloned *cpxR* gene restored the invasion phenotype to a level similar to that displayed by the wild-type strain. Thus, the *cpxR* gene is required for invasion of HM605 into C2Bbe1 epithelial cells.

A



B

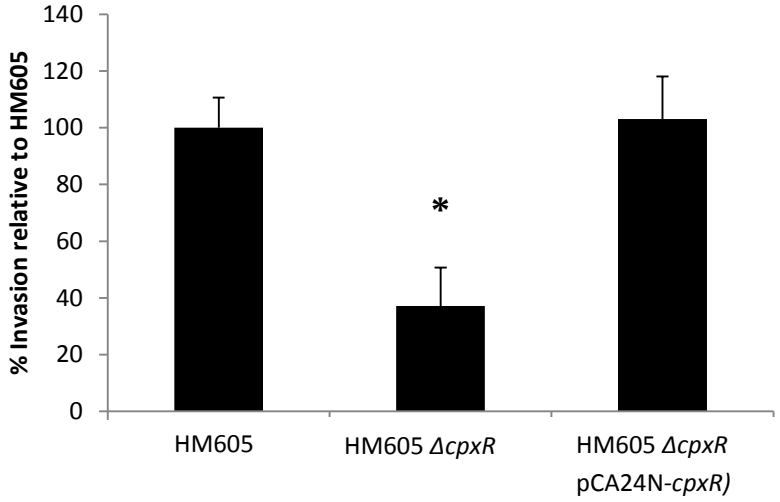


Figure 4.6: A) Cell-association and B) invasion of HM605 and HM605 Δ*cpxR* into epithelial cells. (A) The results are expressed as levels of cell-associated bacteria (adherent plus intracellular) relative to those obtained for wild-type strain HM605, taken as 100%. (B) The *cpxR* gene is reduced in its ability to invade but is not reduced in adherence. The results are expressed as levels of intracellular bacteria/ associated bacteria relative to those obtained for wild-type strain HM605, taken as 100%. For analysis of the significance of differences in association and invasion into C2Bbe1 epithelial cells, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

4.3.5 Motility is reduced in HM605*ΔcpxR*

Numerous studies have implicated flagella-mediated motility in the adherence and invasive abilities of AIEC strain LF82 (Barnich *et al.*, 2003; Eaves-Pyles, 2008). In LF82, flagella have been shown to play a direct role in the adhesion process. Moreover, in addition to down-regulating type 1 fimbriae expression, flagella also play an undefined role in LF82 invasion of epithelial cells (Barnich *et al.*, 2003). Due to the important role of flagella in the pathogenesis of LF82 and in order to examine a possible role of flagella in the invasiveness of HM605 *ΔcpxR::*Km, motility was assessed on swim agar plates. Mutants in *cpxR* were also constructed in *E. coli* strains MG1655 and BW25113 using P1 transduction in order to ascertain if CpxR regulates motility in other *E. coli* strains. Overnight cultures were diluted to and OD_{600} =1.0 in fresh LB and 5µl of the diluted culture was spotted into the 0.3% w/v swim motility agar plate. The plates were incubated for 24h at 37C and the results showed that motility was reduced in the HM605 *ΔcpxR* mutant (see fig 4.7). The halo size in millimetre (mm) of the *ΔcpxR* mutant (6.7mm ± 1.2) was significantly reduced ($P \leq 0.05$) compared to the wildtype (14.3mm ± 1.5) (see fig 4.7A). Moreover, motility in the *ΔcpxR* mutant was successfully restored through the expression of *cpxR in trans* (13.3mm ± 0.6) (see fig 4.7).

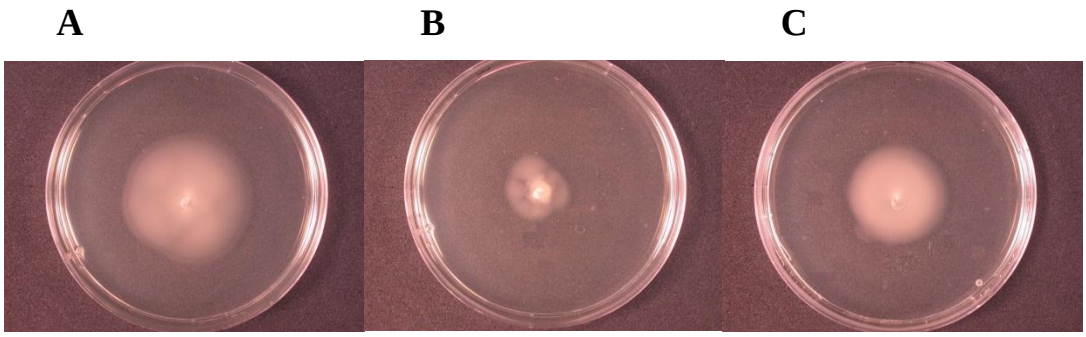


Figure 4.7: Swim motility on 0.3% w/v agar of A) HM605 and B) HM605Δ*cpxR* and C) HM605Δ*cpxR* transformed with cloned *cpxR* gene (pCA24N). Motility is reduced in HM605Δ*cpxR*. Motility was visualized on a 0.3% agar plate as a halo of diffusion of bacteria around the primary inoculum after 24 h at 37°C

In addition, deletion of *cpxR* in both MG1655 (see fig 4.8) and BW25113 (see fig 4.8) dramatically reduced motility similar to what is observed in AIEC HM605.

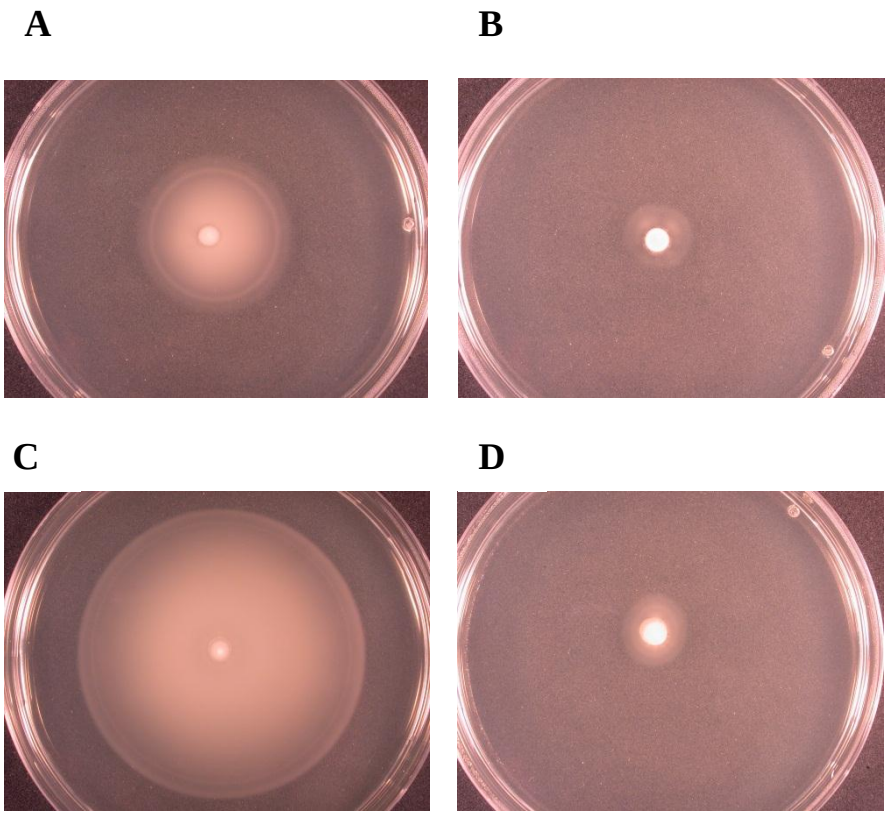


Figure 4.8: Swim motility on 0.3% w/v agar. A) MG1655 and B) MG1655Δ*cpxR*: Km C) BW25113 and D) BW25113Δ*cpxR*::Km. Motility is reduced in HM605Δ*cpxR*. Motility was visualized on a 0.3% agar plate as a halo of diffusion of bacteria around the primary inoculum after 24 h at 37°C

The combined results from these experiments suggest that the CpxA/R TCS positively regulates motility in *E. coli*. Furthermore, as flagella are important for invasion by LF82 it implies that the invasion defect seen in HM605 Δ*cpxR* could be the result of defective flagella synthesis.

4.3.5.1 Identification and characterization of non-motile mutants in HM605

In order to establish whether there was a role for motility in the invasion of C2Bbe1 epithelial cells by HM605 it was decided to screen a transposon mutant library for mutants that were defective in motility. A Tn5 mutant library in HM605, consisting

of 5,568 mutants, was previously created using the EZ-Tn5 transposome®, according to the manufacturers protocol. To facilitate high-throughput analysis, the mutant library was grown in 96-well polystyrene plates (Genetix®) and hand replicated onto Q-trays (Genetix®) containing 250ml of 0.3% swim motility agar (see section 2.9.1) using a 96-well pin replicator. Plates were incubated overnight at 37°C and motility was examined visually. Colonies showing reduced motility were further examined in secondary and tertiary screens. It is important to note that motility in HM605 is very poor and quite difficult to reproduce. Significant heterogeneity in motility is observed in HM605.

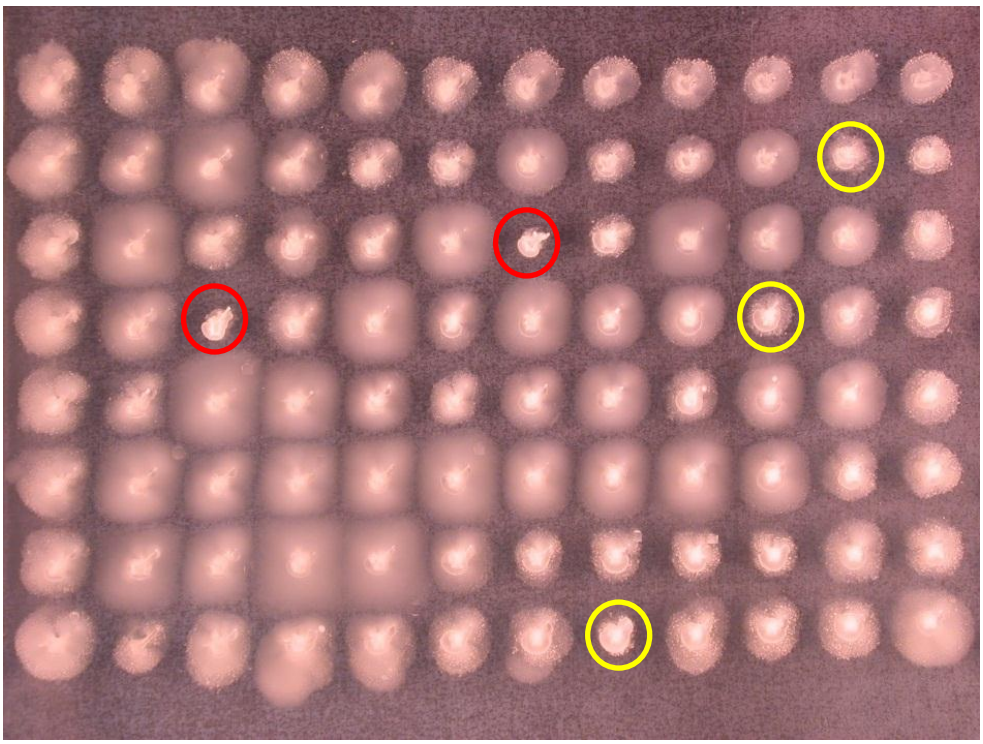


Figure 4.9: An example of a plate from the primary screen of the HM605 Tn5 mutant library screened for swimming motility on 0.3% (w/v) agar. Colonies surrounded by red halo indicate examples of motility minus mutants that were selected for further characterisation. Colonies surrounded by yellow halo indicate examples of colonies showing small halo of diffusion. Only colonies showing a complete reduction in motility were selected (red halo). Motility was visualized on a 0.3% agar plate as a halo of diffusion of bacteria around the primary inoculum after 24 h at 37°C.

Following further screens, a total of 3 non-motile mutants were identified showing a reproducible lack of motility (see table 4.2). Genomic DNA was extracted from all 3 of the these mutants and transposon insertion sites were determined by arbitrarily

primed PCR (see section 2.13). Amplicons were subsequently purified and sequenced and compared to sequences in the NCBI database using a BLAST search.

Table 4.2: The insertion site of the Tn5 for each mutant and the identification of the disrupted gene

Mutant ID in library	Tn5 insertion	Gene disrupted
#37:H8	235544 bp	*flgG*
#10:C2	238530 bp	*flgD*
#29:D8	4366270 bp	*fliH*

Mutant #37:H8 was found to have a Tn5 insertion into codon 112 out of 261 for *flgG* which encodes the flagellar component of cell-distal portion of basal-body rod. FlgG is one of four proteins that comprise the rod section of the basal-body assembly of the flagellar motor (Jones *et al.*, 1990). The basal body spans the cytoplasmic membrane, the peptidoglycan layer, and the outer membrane. A series of rings are linked by a rod to make of the structure of a basal body lying at the base of the flagellar structure. The basal body functions to anchor the flagellum in the cytoplasmic membrane and functions as a rotor during movement (Jones *et al.*, 1990). FlgB, FlgC, FlgF, and FlgG are four proteins that comprise the rod section of the basal-body assembly of the flagellar motor (Saijo-Hamano *et al.*, 2004).

The Tn5 insertion mutant #10:C2 was found to be in codon 121 out of 229 for *flgD*, a gene encoding the flagellar protein FlgD which is reported to act as a scaffold where assembly of hook proteins takes place. Immunoelectron microscopy shows that once the rod is completed, FlgD is added to its end, allowing assembly of hook proteins. Transport of FlgD occurs via the type III flagellar export apparatus. Once the hook is formed, the hook-filament junction proteins FlgK are added to the end and FlgD dissociates (Ohnishi *et al.*, 1994). In *S. enterica* serovar Typhimurium mutants in *flgD* are non-motile (Ohnishi *et al.*, 1994).

Finally, the insertion site of the Tn5 mutant #29:D8 was found to be in codon 136 out of 229 for *fliH* which encodes for the protein FliH, a cytoplasmic protein which exists as a dimer in solution and forms a stable heterotrimeric complex with FliI, inhibiting its ATPase activity (Minamino & MacNab, 2000). FliH is a soluble protein component of the flagellar export apparatus, a type III secretion system that functions in the export of several components of the flagellum across the cytoplasmic membrane into the channel of the flagellum for assembly (Minamino & MacNab, 2000).

4.3.5.2 Flagella are not required for invasion of AIEC HM605

The flagellar mutants were next examined for their ability to adhere to, and invade, C2Bbe1 epithelial cells. All of the motility mutants could adhere to, and invade, C2Bbe1 epithelial cells as well as the wild type (see fig 4.10). Only the *cpxR* mutant was significantly reduced in its ability to invade the epithelial cells. These findings indicate that the invasion defect observed in the Δ*cpxR* mutant is independent of flagella.

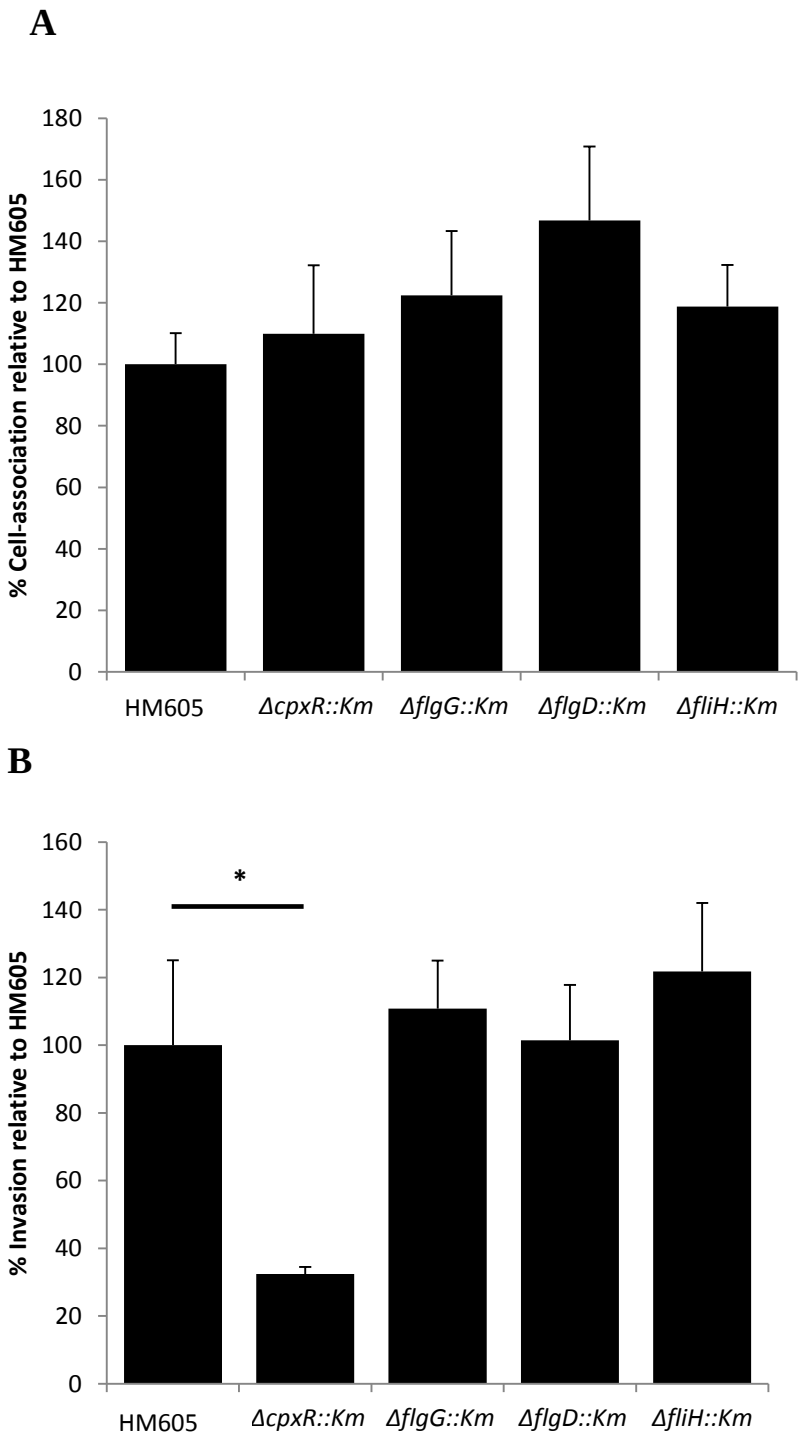


Figure 4.10: Motility is not required for (A) association with, or (B) invasion of, epithelial cells. . (A) Cell-associated bacteria were quantified after a 3-h infection period. The results are expressed as levels of cell-associated bacteria (adherent plus intracellular) relative to those obtained for wild-type strain HM605, taken as 100%. (B) Bacterial invasion was determined after gentamicin treatment for an additional 1 h. The *cpxR* gene is reduced in its ability to invade whereas no other mutant is affected. The results are expressed as levels of intracellular bacteria/associated bacteria relative to those obtained for wild-type strain HM605, taken as 100%. For analysis of the significance of differences in association and invasion into C2Bbe1 epithelial cells, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

4.3.6 HM605 does not produce Type 1 fimbriae

Type 1 fimbriae are the most common filamentous bacterial appendages of *E. coli* and many Gram-negative bacteria and these appendages are major facilitators of host cell invasion by UPEC (Boudeau *et al.*, 2001). The distal part of the tip of type 1 fimbriae contains the mannose-binding adhesin FimH which can attach to mannose moieties of the uroplakin receptors that coat transitional epithelial cells and allow invasion (Jones *et al.*, 1995; Mulvey *et al.*, 1998; Sauer *et al.*, 2000). Furthermore, type 1 fimbriae have previously been reported to be required for epithelial cell invasion of AIEC strain LF82 (Barnich *et al.*, 2003; Boudeau *et al.*, 2001). Two non-invasive type 1 fimbrial mutants in AIEC LF82, which harboured an insertion within the *fimI* and *fimF* genes, still adhered but had lost the ability to induce host cell membrane elongations at the sites of contact with the epithelial cells (Boudeau *et al.*, 2001). As type 1 fimbriae are known invasion factors of LF82, the next stage was to examine the potential role of type 1 fimbriae in the invasion of HM605.

The presence of functional type 1 fimbriae on the bacterial surface was assessed by the ability of the bacteria to aggregate yeast cells via binding to D-mannose residues (Barnich *et al.*, 2003). Commercial baker's yeast (*Saccharomyces cerevisiae*) was suspended in PBS (4 mg/ml dry weight). Equal amounts of yeast suspension and bacterial suspension were mixed in a 24-well polystyrene plate (Sartstedt®) and aggregation was monitored visually. The commensal *E. coli* MG1655 was used as a positive control. MG1655 strongly aggregated yeast cells whilst, HM605 and the Δ*cpxR*::Km mutant were both unable to aggregate yeast cells (fig 4.11). Therefore HM605 does not produce type 1 fimbriae.

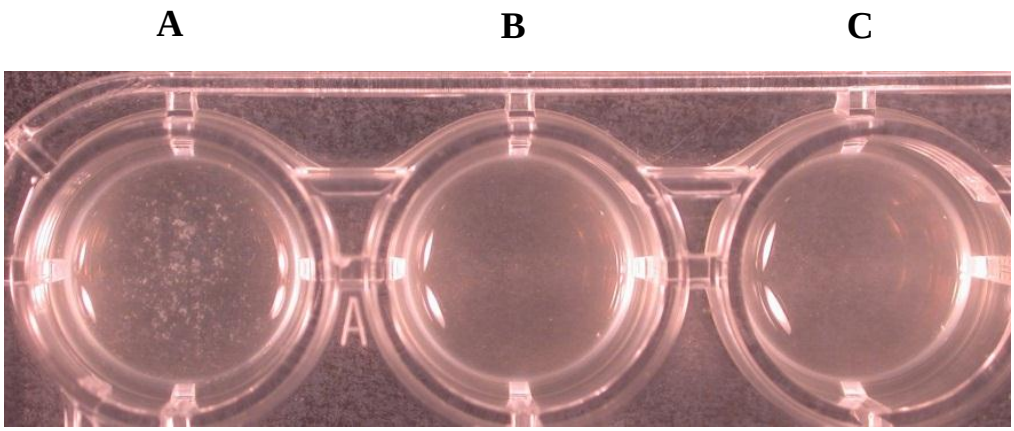


Figure 4.11: HM605 does not produce Type 1 fimbriae. A) MG1655 strongly aggregated yeast. B) HM605 and C) HM605 Δ*cpxR* were unable to aggregate yeast.

In *E. coli* the expression of type 1 pili expression is mediated by a process called phase variation, in which the bacteria can switch between piliated and non-piliated states under the control of a 314-bp invertible DNA element, the *fim* switch (*fimS*), that is located upstream of the fim operon, encoding the type 1 fimbriae and containing the promoter of the fim operon (see section 2.19) (Barnich *et al.*, 2003; Smith & Dorman, 1999). Therefore the orientation of the fim switch determines whether the fim operon is transcribed or not (see fig 2.2).

The genome sequence of HM605 revealed that this bacterium does contain a fim operon. However there is an insertion within the coding region of an upstream gene called *fimB*. FimB is a recombinase that catalyses the site-specific recombination required for inversion of the fim switch (Klemm, 1986; McClain *et al.*, 1991). FimB is responsible for both OFF-to-ON and ON-to-OFF switching at equal rates (see section 2.19) (Klemm, 1986). In contrast, *fimE* which encodes a second recombinase is only responsible for only ON-to OFF switching (Stentebjerg-Olesen *et al.*, 1999). In accordance with the reported directionality of the FimB and FimE recombinase activities, an insertion in *fimB* would readily account for the off orientation of the phase switch and the Fim-negative status of the cells. To confirm that the fim switch was locked OFF in HM605 and that the orientation of the switch was not affected by the *cpxR* mutation the orientation of the fim switch was determined using a PCR-based assay. (Smith & Dorman, 1999). This method exploited a unique *BstUI* restriction site in the *fim* switch that results in restriction fragment length dimorphism among *BstUI*-digested PCR products that can distinguish between switches that are in the ON or OFF orientation. Using this assay, bacteria with the fim switch in the ON orientation would yield 2 DNA fragments of 433 and 293 bp, whereas bacteria with the fim switch in the OFF orientation would yield fragments of 539 and 187 bp (see section 2.18 fig 2.2). For MG1655, as expected, all 4 bands were detected suggesting that within the population of bacteria tested the fim switch could be detected in both the ON and OFF orientation, typical of phase variation (see fig 4.12). However, HM605 and HM605 Δ*cpxR* produced the products associated exclusively with the OFF orientation confirming the fim switch is locked OFF in HM605 and the *cpxR* mutant resulting in no expression of the fim operon. Therefore Type 1 fimbriae are unlikely to play a role in the invasion of epithelial cells by HM605.

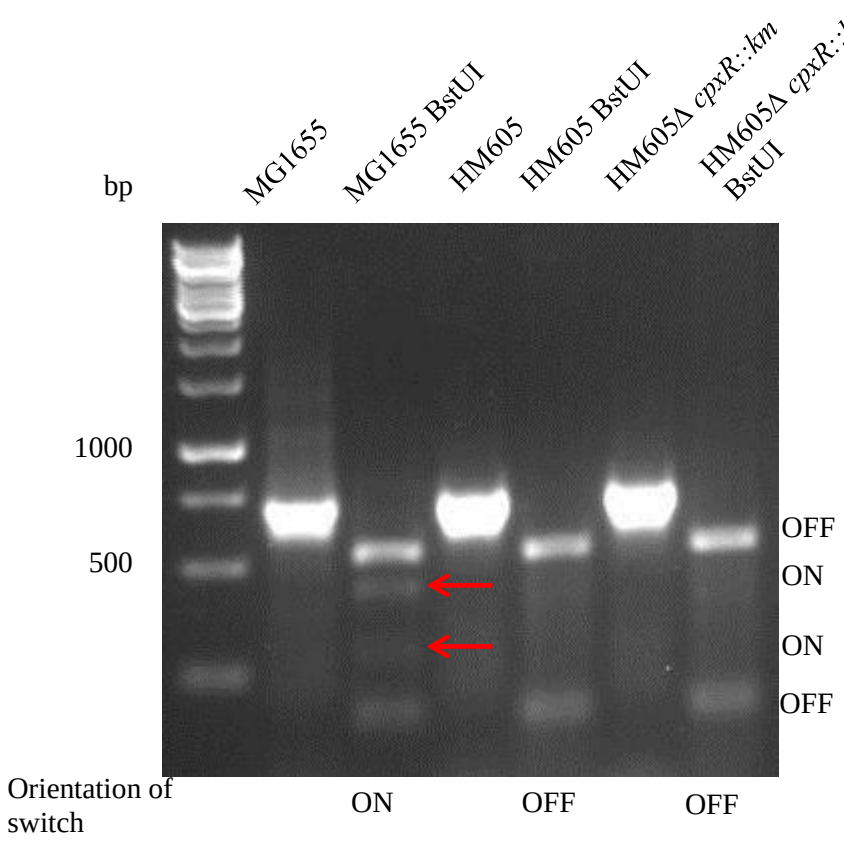


Figure 4.12: Determination by PCR analysis of the invertible element orientation of the *fim* operon. 433-bp and 293-bp products revealed the phase-ON orientation, and 750-bp and 187-bp products revealed the phase-OFF orientation of the invertible element. A mixture was observed for MG1655 indicating the expression of type 1fimbriae. HM605 and HM605 Δ*cpxR::*km revealed the phase-OFF orientation indicating a lack of type fimbrial expression.

4.3.7 σ^E (RpoE) expression is up-regulated in HM605Δ*cpxR*

The observed lack of involvement of previously described invasion factors led to the question of whether the *cpxR* mutant was accumulating misfolded outer membrane proteins leading to alterations in the expression of potential outer membrane invasion factors. As previously stated, the main function of the CpxA/R TCS is to respond to damage to the cell envelope via the production of proteases and folding catalysts (Dorel *et al.*, 2006; Price & Raivio, 2009). In addition to the CpxA/R TCS, the σ^E (RpoE) envelope stress response pathway is also activated by events or mutations that results in damage to the outer membrane (Mecsas *et al.*, 1993; Raivio, 2005). Accumulation of misfolded outer membrane proteins (OMP) leads to the proteolytic cleavage of the membrane-bound antisigma factor RseA, which releases σ^E into the cytoplasm resulting in the transcriptional activation of a set of genes that includes many that are involved in OMP folding and outer membrane biogenesis (Raivio, 2005). One possibility is that membrane damage due to the *cpxR* mutation may result in the misfolding of an outer membrane protein that might be involved in invasion. Membrane damage will result in activation of σ^E activity and, as the *rpoE* gene is auto-regulated, activation of σ^E activity will result in an increase in *rpoE* (Price & Raivio, 2009; Raivio, 2005). Therefore, *rpoE* expression can be used as a reporter of possible outer membrane folding problems in the *cpxR* mutant. A transcriptional *rpoE*-gfp fusion was used to analyse the expression of *rpoE* in wildtype HM605 and the *cpxR* mutant. The data produced from monitoring growth and GFP indicate that the expression of *rpoE* is significantly up-regulated in the *cpxR* mutant compared to the wild-type (fig 4.13). In addition, Figure 4.13C shows the activity of the rpoE promoter (calculated as $[\Delta GFP/\Delta t]/\Delta OD$) reached a maximal level after approximately 2h in both strains. This indicates that growth is associated with an increase *rpoE* activity probably due to the requirement to fold OMPs as the cells grow and divide during exponential growth. However; there was a significantly higher level of *rpoE* promoter activity in the *cpxR* mutant compared to the wild-type confirming that there is likely to be an accumulation of mis-folded OMPs in the *cpxR* mutant compared to wild-type.

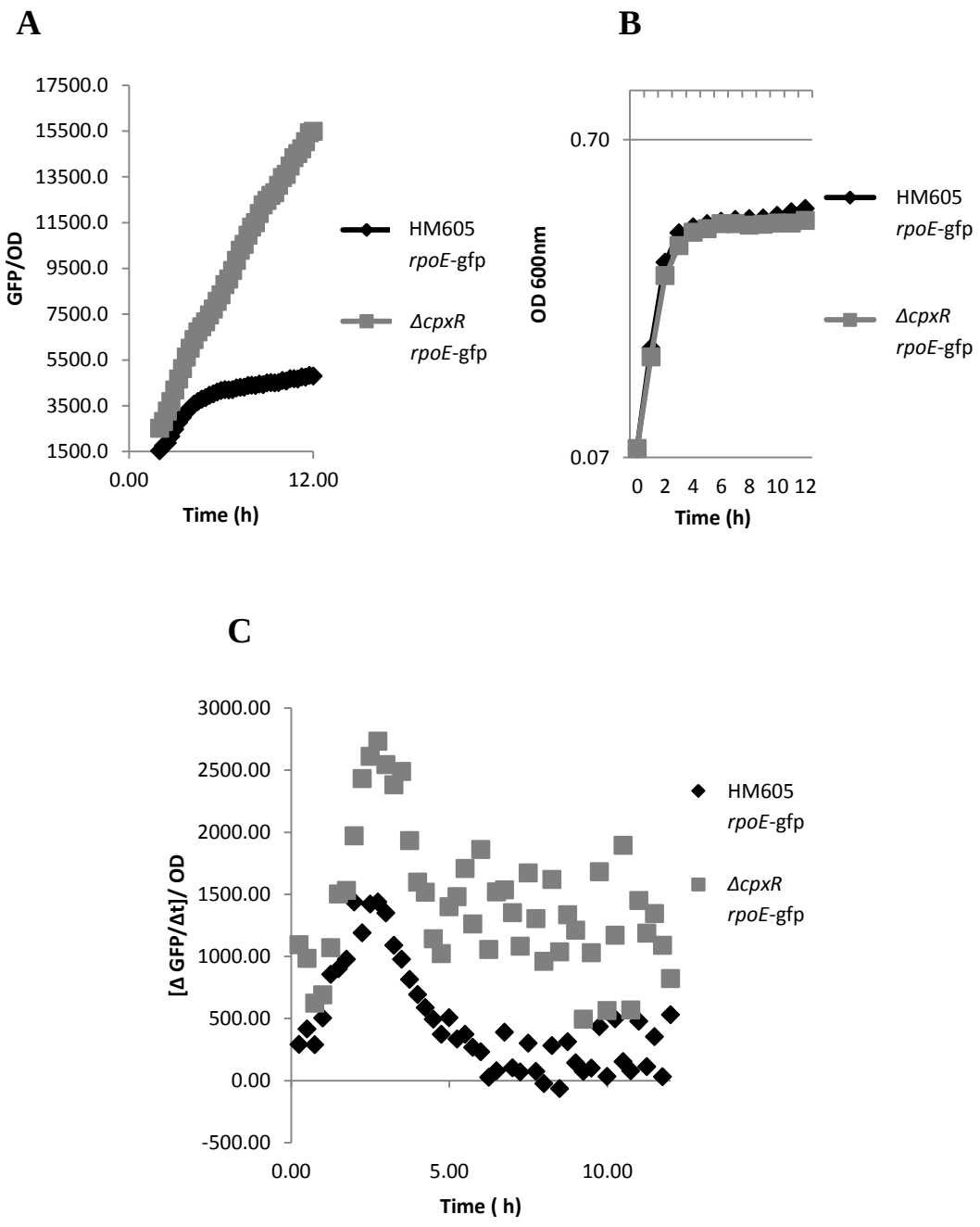


Figure 4.13: Expression of *rpoE* in HM605 and HM605 *ΔcpxR*. A) The expression of *rpoE* is significantly up-regulated in the *cpxR* mutant throughout growth. Results are expressed as the amount of GFP expressed relative to growth (GFP/OD). B) Growth of wildtype HM605 and the *cpxR* mutant in LB. C) Promoter activity of *rpoE*-gfp. Each strain was represented by 3 individual cultures and each value is the mean of the 3 individual cultures.

4.3.7.1 Overproduction of cell envelope maintenance proteins DsbA and DegP in HM605*ΔcpxR* restore RpoE expression to wildtype levels

In the previous section it was shown that misfolded OMPs are accumulating in the *cpxR* mutant and it is possible that these OMPs are required for invasion of epithelial cells. DegP and DsbA are periplasmic proteins produced by the cell in response to misfolded extracellular proteins. The genes encoding these proteins are positively regulated by the Cpx A/R TCS and it is likely that the accumulation of misfolded OMPs in the *cpxR* mutant is due to a reduction in the production of one, or both, of these proteins. It was decided to determine whether OMP stress in the *cpxR* mutant could be alleviated by the heterologous production of either DegP or DsbA and if this correlated with a restoration of invasion. The ASKA library contains 85% of *E. coli* K-12 ORFs cloned into plasmids under the control of the $P_{T5\text{-}lac}$ promoter that can be activated by IPTG (Kitagawa *et al.,* 2005). Plasmids encoding the *dsbA* and *degP* genes were transformed into HM605 (*rpoE*-gfp) or HM605*ΔcpxR* (*rpoE*-gfp) and growth and fluorescence were monitored over time in the presence of IPTG (see fig 4.14).

Overproducing either DsbA or DegP in the *cpxR* mutant restored *rpoE* expression and promoter activity to wildtype levels indicating that overproduction of these periplasmic proteins could alleviate the OMP stress observed in the cpxR mutant (fig 4.14). Furthermore, DsbA or DegP did not rescue the invasion defect associated with the *cpxR* mutant. Therefore the OMP stress induced by the *cpxR* mutation can be rescued by the overproduction of membrane chaperone/proteases although invasion appears to be independent of this OMP stress or requires a protein(s) that is not a substrate for either DegP or DsbA (fig 4.15).

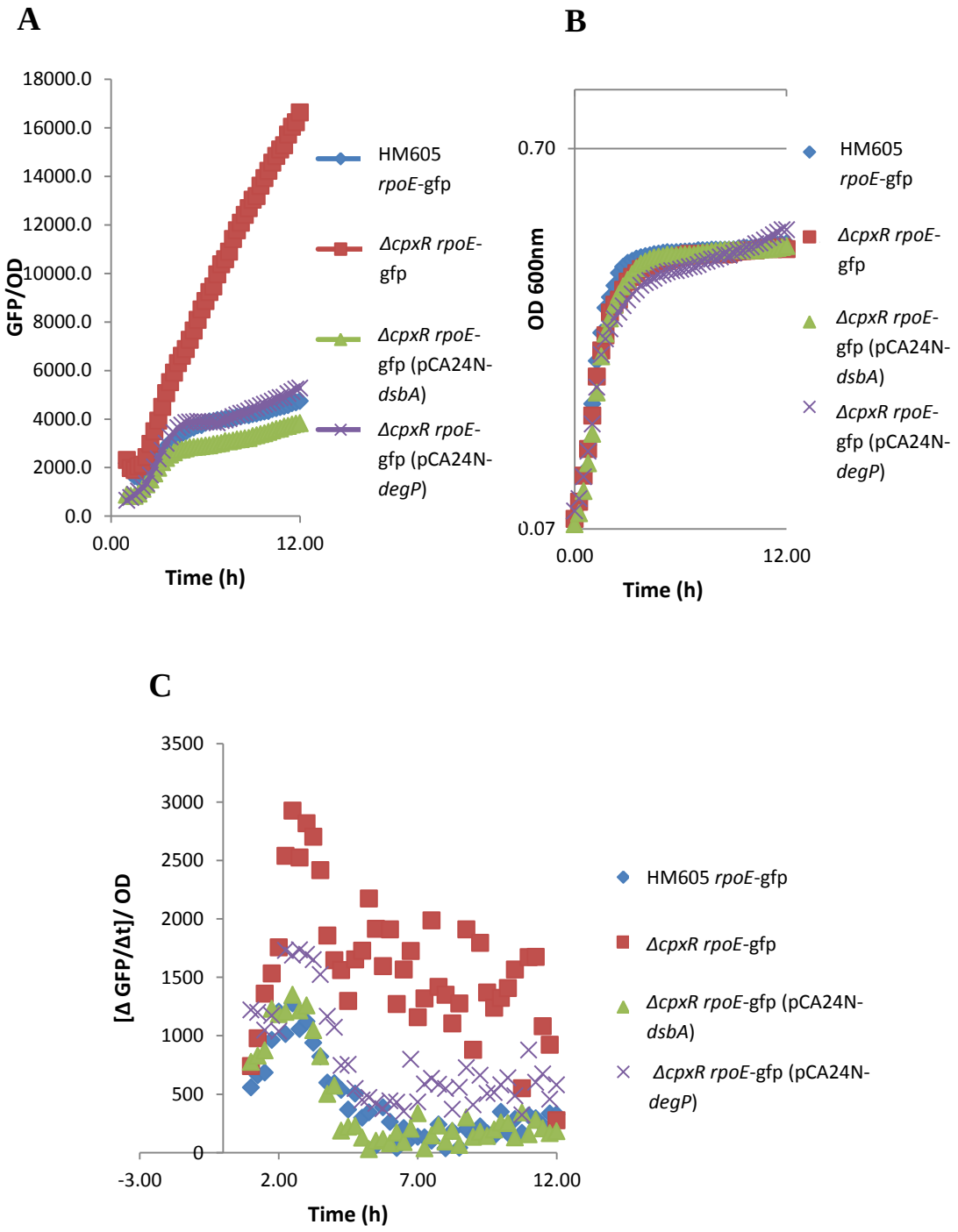


Figure 4.14: Overproduction of DsbA and DegP in HM605*ΔcpxR* restores the expression of *rpoE* to wildtype levels. A) GFP/OD600 showing the levels of GFP expression. The expression of *rpoE* is significantly up-regulated in the *cpxR* mutant. However, *rpoE* expression was restored to wildtype levels in the *cpxR* mutant when DsbA and DegP were overproduced. Results are expressed as the amount of GFP expressed relative to growth (GFP/OD). B) Growth of all strains in LB. C) Promoter activity of *rpoE*-gfp. Promoter activity reached its maximal level at approximately 2h in all strains however; there was a significantly higher induction in the *cpxR* mutant which was reduced when both DsbA and DegP were overproduced. Each strain was represented by 3 individual cultures.

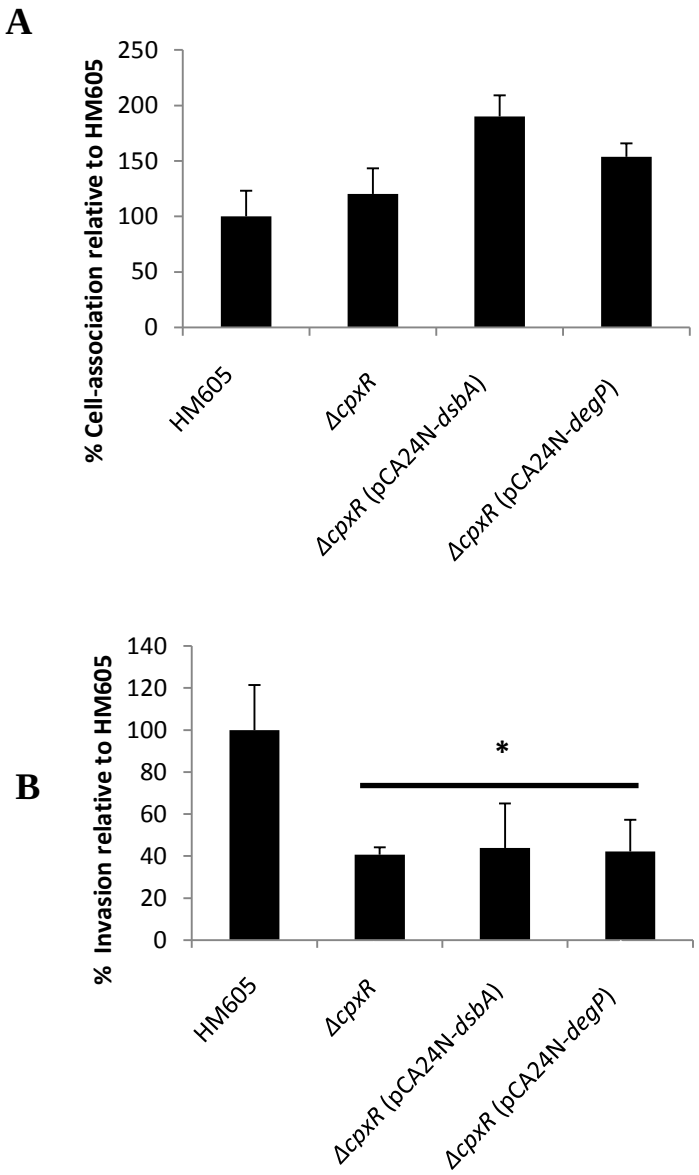


Figure 4.15: Phenotype of HM605Δ*cpxR* (pCA24N-*dsbA*) and HM605Δ*cpxR* (pCA24N-*degP*) in C2Bbe1 epithelial cells. (A) Adhesion to C2Bbe1 epithelial cells. Cell-associated bacteria were quantified after a 3-h infection period. The results are expressed as levels of cell-associated bacteria (adherent plus intracellular) relative to those obtained for wild-type strain HM605, taken as 100%. (B) Bacterial invasion of C2Bbe1 epithelial cells was determined after gentamicin treatment for an additional 1 h. Overproduction of DsbA and DegP in the *cpxR* mutant did not restore the invasion phenotype. The results are expressed as levels of intracellular bacteria/associated bacteria relative to those obtained for wild-type strain HM605, taken as 100%. For analysis of the significance of differences in association and invasion into C2Bbe1 epithelial cells, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

4.3.8 Preparing RNA for Microarray

The HM605 *ΔcpxR* mutant, although proficient at attachment, is reduced in its ability to invade C2Bbe1 epithelial cells and this invasion defect is independent of flagella and type 1 fimbriae, known invasion factors in another strain of AIEC called LF82. In order to investigate the role of the CpxA/R TCS in the process of invasion it was decided to characterise the Cpx regulon in HM605 using transcriptomics.

It has been well established that moderate overexpression of the outer membrane lipoprotein NlpE activates the CpxA/R TCS (Otto & Silhavy, 2002; Snyder *et al.*, 1995). Therefore the *nlpE* clone from the ASKA library (in the pCA24N vector and under the control of the $P_{T5\text{-}lac}$ promoter) was transformed into HM605 containing the *ycfS* transcriptional GFP-promoter fusion. Expression of the *ycfS* gene is positively regulated by the Cpx pathway in HM605 (see fig 4.5) and the induction of the CpxA/R TCS by NlpE can therefore be monitored by examining the GFP expression of HM605 (*ycfS*-gfp). Four concentrations of IPTG were tested (1µM, 10µM, 100µM and 1mM) to identify a concentration that allows induction without affecting bacterial growth. Overnight cultures of the test strains were diluted to OD_{600} 0.05 in 5mls of LB (Km Cm) and each strain was represented by 3 individual cultures. Cultures were grown until OD_{600} 0.2 and either 1µM, 10µM, 100µM or 1mM IPTG was added to overproduce NlpE. OD_{600} and GFP fluorescence (Ex.485/20 and Em.528/20) readings were taken every 15 minutes over a period of 24 hours. In wildtype HM605 the overproduction of NlpE with all concentrations of IPTG activates the CpxA/R TCS as significant increases in fluorescence were observed indicating an increase in the expression of the *ycfS*-gfp promoter fusion (see fig 4.16). However, all concentrations of IPTG, apart from 10µM, affected the growth of HM605 and, therefore, it was decided to use 10µM IPTG in future transcriptomic experiments. Induction of *ycfS*-gfp expression was confirmed to be dependent on *cpxR*.

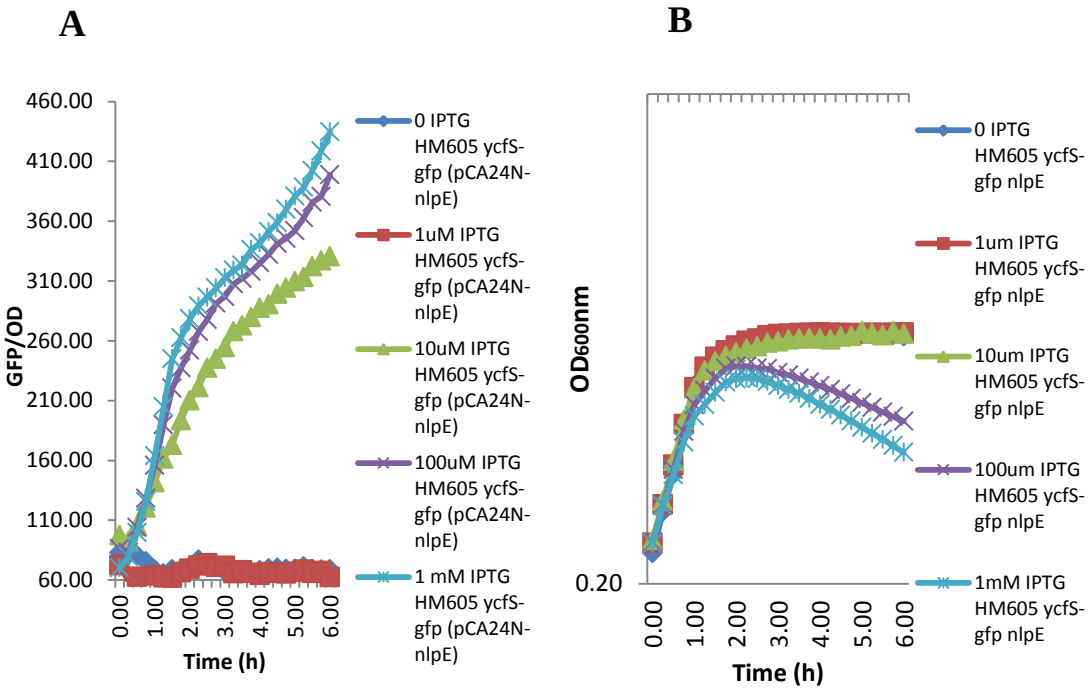


Figure 4.16: Activation of Cpx pathway in HM605 *ycfS*-gfp (pCA24N-*nlpE*) using various concentrations of IPTG A) GFP/OD600 showing the levels of GFP expression. The expression of *ycfS* is significantly up-regulated at IPTG concentrations above 1µM. Results are expressed as the amount of GFP expressed relative to growth (GFP/OD). B) Growth of all strains in LB with various concentrations of IPTG. 10 µM was the only concentration that activated the pathway but didn't affect growth. Each strain was represented by 3 individual cultures.

The research group of Prof Tracy Raivio (University of Alberta) is currently looking at the CpxA/R regulon in *E. coli* K-12 using the ASKA pCA24N-*nlpE* clone and inducing expression of the *nlpE* for 25-30 minutes during the mid-exponential phase of growth before isolating RNA (Tracy Raivio, personal communication). Therefore, in order to facilitate downstream comparative studies, it was decided to replicate these conditions as closely as possible. The growth and fluorescence of HM605Δ*cpxR ycfS*-gfp (pCA24N-*nlpE*) and HM605 *ycfS*-gfp (pCA24N-*nlpE*) was measured after 30 minutes induction of pCA24N-*nlpE* with 10µM IPTG and this confirmed that the CpxA/R TCS is activated under these conditions (see fig 4.17).

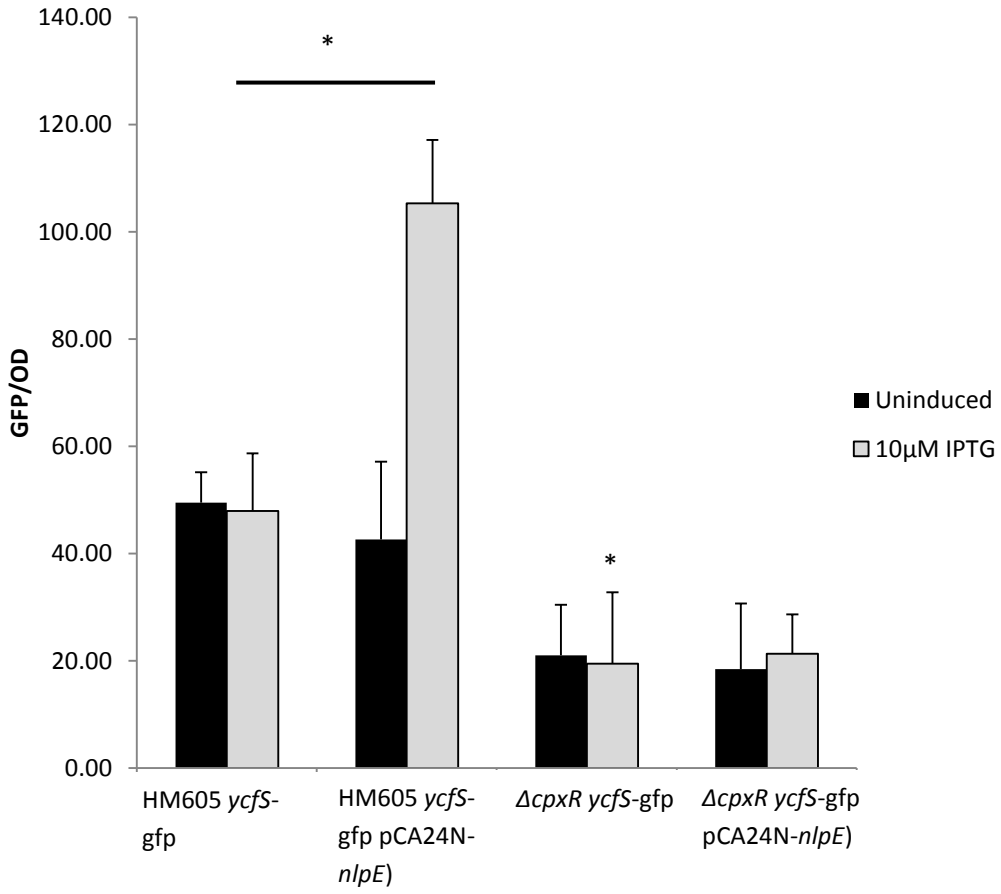


Figure 4.17: GFP/OD_{600}nm showing the levels of GFP expression using *ycfS*-gfp reporter gene after 30 minutes. In the wildtype, activation of the wildtype cpxA/R TCS using NlpE shows a significant increase in *ycfS*-GFP expression as the pathway is activated whereas in the *cpxR* mutant little to no increase in GFP expression is observed as the pathway has been disrupted. Each strain was represented by 3 individual cultures. For analysis of the significance of differences in expression, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

Therefore, using these conditions, microarray RNA samples were prepared from 3 individual cultures of HM605-*ycfS*-gfp (pCA24N-*nlpE*) and HM605Δ*cpxR*-*ycfS*-gfp (pCA24N-*nlpE*). Briefly, overnight cultures were diluted to an OD_{600} of 0.05 in 50ml of fresh LB broth (with appropriate antibiotics) in 200ml conical flasks. When the cultures had reached OD600=0.5, 10µM IPTG was added for 30 min, at which time three 4ml samples were harvested. The RNA was stabilized using Qiagen RNA protect before pellets were frozen at -80^{o}C. RNA was extracted using Qiagen RNeasy kit (see section 2.34). The RNA was treated with DNase (Ambion) and after DNase treatment, the RNA was checked by running a sample through a 1.5% (w/v) agarose 1xTBE gel. A PCR was performed using a sample DNase treated RNA as template and *cpxP* primers (*cpxP* Fw(XhoI) and *cpxP* Rv(BamHI)) to confirm the absence of DNA. The RNA was quantified and the concentrations are summarised in Table 4.3.

Table 4.3: RNA concentrations of each sample

Strains	A260/280	A260/230	ng / µl	Volume in tube (µl)	µg / sample
HM605-*ycfS*-gfp (pCA24N-nlpE) 1	2.11	1.96	704.7	35	24.66
HM605-*ycfS*-gfp (pCA24N-nlpE) 2	2.11	2.12	1198	44	52.71
HM605-*ycfS*-gfp (pCA24N-nlpE) 3	2.09	2.14	1307.1	44	57.52
HM605Δ*cpxR* -*ycfS*-gfp (pCA24N-nlpE) 1	2.02	1.98	704.2	35	24.64
HM605Δ*cpxR* -*ycfS*-gfp (pCA24N-nlpE) 2	2.06	1.97	682.5	40	27.30
HM605Δ*cpxR* -*ycfS*-gfp (pCA24N-nlpE) 3	2.09	1.97	580.2	40	23.21

The RNA samples were shipped on dry ice to Roche NimbleGen™ High-definition genomics where hybridisations to *E. coli* HM605 tiled arrays were performed. The output data from the hybridisations was processed by NimbleGen using their stringent criteria for normalised calls. The data was returned in the form of original data and normalised calls that could then be further analysed.

4.3.9 Microarray analysis

The tiling array was produced based on the genome sequence of HM605 (Clarke et al., 2011). The spacing in the tiling array is 141bp and therefore there are >36,000 70-mer oligonucleotides spanning the genome sequence. Microarray data was analysed by Mr Ian O Neill, Department of Microbiology, University College Cork (personal communication) using the ANAIS online tool (http://anais.versailles.inra.fr/) (Simon & Biot, 2010). Prior to differential expression analysis, each probe on the array was matched to annotated genes on the HM605 genome using custom perl scripts. Where multiple probes matched a single gene, the median value of all probes was taken. Differential expression analysis between wild type HM605 and the *ΔcpxR* mutant was performed on all HM605 genes using the ANAIS tool. Raw probe reads were normalised using RMA background correction and quantile normalisation. Statistical significance was evaluated using one-way ANOVA and p-values were adjusted for multiple comparisons using the Benjamini and Hochberg method.

Table 4.4: The top 40 genes found to be significantly down-regulated in *ΔcpxR* compared to WT. Significant fold changes determined using the Students T-test and those genes previously shown within literature to be regulated by the Cpx A/R TCS are in bold.

Locus tag	Gene Name	Description	↓ Fold change	P value
HM605_2086	***cpxP***	Periplasmic repressor	-215.19	3E-04
HM605_0238	***ycfS***	promoter-gfp fusion	-119.80	2E-03
HM605_4614	***spy***	periplasmic protein	-44.02	3E-03
HM605_4896	***yncJ***	predicted signal peptide	-30.87	5E-04
HM605_2966	***yqjB (mzrA)***	modulator of EnvZ/OmpR)	-26.98	1E-02
HM605_3823	*yfdX*	hypothetical protein	-17.23	2E-02
HM605_4569	***yeaI***	putative diguanylate cyclase	-16.06	8E-03
HM605_4781	*ynfD*	ynfD	-13.82	2E-03
HM605_3701	*sinI/sivI*	Invasion –like protein	-13.36	2E-02
HM605_2961	*yqjK*	hypothetical protein	-12.61	1E-02

HM605_4539	*yoaE*	putative membrane protein fused with conserved domain	-12.60	2E-03
HM605_0080	*chaA*	calcium/sodium:proton antiporter	-11.97	2E-03
HM605_4828	*ydeQ*	fimbrial operon	-11.63	8E-03
HM605_2967	***yqjA***	putative inner membrane protein (co-transcribed with ybjB (mzrA))	-11.28	3E-03
HM605_1398	*gnsB*	hypothetical protein	-10.76	1E-02
HM605_2702	*nirC*	nitrite transporter	-10.09	5E-03
HM605_2099	*rhaA*	L-rhamnose isomerase	-9.28	2E-03
HM605_2088	***cpxA***	Sensor kinase	-9.23	4E-03
HM605_4009	*yfaD*	hypothetical protein	-9.22	9E-02
HM605_0814	*ybbP*	putative ABC transporter permease	-8.94	5E-03
HM605_4768	*mdtI*	multidrug efflux system protein	-8.50	1E-02
HM605_3538	*ygaU*	LysM domain/BON superfamily protein	-8.49	9E-04
HM605_4829	*ydeP*	putative oxidoreductase (downstream from HM605_4828 above)	-8.23	5E-03
HM605_4506	***yebE***	conserved hypothetical protein; putative inner membrane protein	-8.12	1E-02
HM605_1946	*ubiC*	Chorismate lyase-like	-8.10	1E-02
HM605_4524	***htpX***	heat shock protein	-7.87	1E-03
HM605_4013	*glpA*	sn-glycerol-3-phosphate dehydrogenase subunit A	-7.84	3E-02
HM605_3700	*sivH/sinH*	putative intimin ((attaching and effacing protein) or invasin protein (sivH-like)	-7.51	1E-02
HM605_4509	*yobB*	hypothetical protein	-7.51	1E-02
HM605_1159	*cdaR*	carbohydrate diacid transcriptional activator	-7.42	1E-02
HM605_4090	*fruB*	bifunctional fructose-specific PTS IIA/HPr protein	-7.39	2E-02
HM605_1345	*slt*	lytic murein transglycosylase	-7.23	5E-03
HM605_0815	*ybbA*	putative ABC transporter ATP-binding protein	-7.08	2E-02
HM605_1399	*cspl*	cold shock protein; Qin prophage	-7.08	2E-03
HM605_4190	HM605_4190	potential phage operon	-6.70	1E-02

HM605_3539	*csiR*	DNA-binding transcriptional regulator	-6.58	1E-02
HM605_1687	yjeT	Conserved inner membrane protein	-6.31	9E-03
HM605_0376	*tusE*	sulfur transfer protein TusE	-6.29	1E-02

Table 4.5: The top 40 genes found to be significantly up-regulated in *ΔcpxR* compared to WT. Significant fold changes determined using the Students T-test.

Locus tag	Gene Name	Description	↑ Fold change	P value
HM605_3107	*glcD*	Glycolate oxidase subunit	80.47	2E-03
HM605_1037	*yagU*	Conserved hypothetical protein; putative inner membrane protein	51.03	5E-04
HM605_3108	*glcE*	Glycolate oxidase FAD binding subunit	38.80	8E-03
HM605_3109	*glcF*	Glycolate oxidase iron-sulfur subunit	23.09	8E-03
HM605_3438	*cysD*	Sulfate adenylyltransferase subunit 2	22.55	8E-03
HM605_2927	*agaB*	N-acetylgalactosamine-specific PTS system transporter subunit IIB	14.59	1E-02
HM605_2103	*yiiE*	Hypothetical protein YiiE	12.34	1E-03
HM605_3110	*glcG*	Hypothetical protein	11.91	2E-03
HM605_2104	*ynfD*	Downstream from formate dehydrog., potential small RNA	10.97	3E-02
HM605_3112	*yghK*	Glycolate transporter	10.37	3E-02
HM605_2532	*hdeA*	Acid-resistance protein	9.15	2E-02
HM605_0328	*pgaA*	N-deacetylated poly-β-1,6-N-acetyl-D-glucosamine outer membrane porin;	9.13	1E-02
HM605_4655	*ppsR (ydiA)*	Regulator of PEP synthase (ppsA))	8.18	1E-02
HM605_1835	*adiY*	DNA-binding transcriptional activator	8.14	1E-02
HM605_3111	*glcB*	Malate synthase G	7.61	4E-03
HM605_2465	*xylF*	D-xylose transporter subunit XylF	7.46	1E-02
HM605_1979	*aceB*	Malate synthase	7.44	1E-02

HM605_0330	*pgaC*	N-glycosyltransferase	7.44	6E-03
HM605_1978	*aceA*	Isocitrate lyase	7.39	1E-02
HM605_0329	*pgaB*	Putative polysaccharide deacetylase associated with biofilm formation; putative lipoprotein	7.32	2E-02
HM605_2533	*hdeB*	Acid-resistance protein	7.19	2E-02
HM605_4850	*bdm*	Biofilm-dependent modulation protein	7.13	6E-02
HM605_2530	*gadE*	DNA-binding transcriptional activator	7.04	1E-02
HM605_1014	*ykgC*	Pyridine nucleotide-disulfide oxidoreductase	7.00	3E-02
HM605_2369	*gltS*	Putative PTS system transcriptional antiterminator	6.94	8E-03
HM605_4729	*tppB*	Putative tripeptide transporter permease	6.72	2E-03
HM605_1024	*yaqV*	Hypothetical protein	6.70	9E-03
HM605_4218	*gpE*	Tail protein E	6.70	1E-02
HM605_3441	*ygbE*	hypothetical protein (part of cysDNC operon)	6.59	2E-02
HM605_2034	*rspA*	putative enolase/dehydratase	6.48	2E-02
HM605_0998	*yahA*	cyclic di-GMP phosphodiesterase	6.47	2E-02
HM605_1025	HM605_1025	Putative NADH-dependent flavin oxidoreductase	6.30	2E-03
HM605_2531	*hdeD*	Acid-resistance membrane protein	6.16	2E-02
HM605_0650	*kdpE*	KDP operon transcriptional regulator protein (KdpE-like)	6.11	3E-02
HM605_4799	*yneJ*	Putative DNA-binding transcriptional regulator HTH family	6.07	9E-03
HM605_0331	*pgaD*	Putative inner membrane protein associated with biofilm formation	6.02	3E-02
HM605_3780	*cysK*	Cysteine synthase A	5.93	2E-02
HM605_3439	*cysN*	Sulfate adenylyltransferase subunit 1	5.82	4E-02
HM605_0332	*phoH*	Hypothetical protein	5.76	6E-02

4.3.9.1 Microarray data

The first striking observation from the transcriptome data was that a large number of genes were up (409 genes) or down (524) regulated in *ΔcpxR*, compared to wildtype, by > 2 fold following the activation of the pathway by NlpE. Interestingly, many of the genes identified are predicted to encode proteins that influence envelope protein folding, acid resistance and functions undefined or unrelated to envelope maintenance. Such an observation has been observed in other Cpx regulon studies (Price & Raivio, 2009). For example, the gene *yqjA* was shown to be expressed at lower levels in the *cpxR* mutant compared to wildtype. YqjA is predicted to be an inner membrane protein and mutations of *yqjA* lead to altered levels of phospholipids in the inner membrane and cell division defects (Price & Raivio, 2009; Thompkins *et al.*, 2008). Similarly, *chaA* which encodes the ionic transporter membrane protein ChaA involved in sodium ion extrusion in *E. coli* at alkaline pH was significantly expressed at lower levels in the *cpxR* mutant compared to wildtype. It is important to note that a large number of genes expressed at lower levels in *ΔcpxR* compared to the wild-type have been previously reported to be controlled by the Cpx pathway, some of which implicated in envelope protein folding functions, *cpxP, cpxA, spy* and *secA* (see table 4.3) (Danese & Silhavy, 1998; Price & Raivio, 2009; Raivio *et al.*, 1999; Raivio *et al.*, 2000). Other genes expressed at lower levels in *ΔcpxR* compared to the wild-type such as *ycfS, yebE* (Price & Raivio, 2009), *yqjB (mzrA)* (Gerken *et al.*, 2009), *yqjA* (Weatherspoon-Griffin *et al.*, 2010), *yncJ* and *yeaI* (Zare *et al.*, 2009) have all also been previously reported to be controlled by the Cpx pathway. The gene *htpX* which encodes the heat shock protein HtpX was shown to be expressed significantly lower in *ΔcpxR* compared to wildtype. Similarly, *yqjB (mzrA)* which encodes the EnvZ/ OmpR modulator MzrA has been previously reported to link the two-component envelope stress response regulators, CpxA/CpxR and EnvZ/OmpR and its expression is reduced in a *cpxR* mutant (Gerken *et al.*, 2009).

An interesting observation was that a number of acid resistance genes were expressed at a significantly higher level in the *cpxR* mutant compared to wildtype, primarily *hdeA, hdeB, hdeD* and *gadE*. The CpxA/R TCS has previously been implicated in resistance to alkaline pH were *cpxR* null mutants show increased sensitivity to alkaline pH (Danese & Silhavy, 1998). However, no reports show

evidence of *cpxR* null mutants displaying a higher resistance to low pH. The results presented here suggest that HM605*ΔcpxR* may be more resistant to acid than wildtype HM605. To test this overnight cultures of the test strains were diluted to OD_{600} 0.05 in 5ml of LB (+ Km for mutants) and each strain was represented by 3 individual cultures. Cultures were grown until OD_{600} 0.5 and following this strains were acid challenged by diluting cultures 1 in 10 into pH 2.5 LB (+ 100mM MES) for 30 minutes. Strains were then serial diluted and plated on to LB agar plates and incubate at 37^{o}C for approximately 24 h. The results from the acid challenge experiment show that HM605*ΔcpxR* was significantly more tolerant to low pH as it was able to survive better at pH 2.5 compared to wildtype HM605 (see fig 4.18). These results support the findings of the microarray where acid resistance genes *hdeA, hdeB, hdeD* and *gadE* are expressed significantly higher in HM605*ΔcpxR* compared to wildtype. In contrast, no difference was observed between MG1655 and MG1655*ΔcpxR*. Both strains showed similar levels of survival at low pH (see fig 4.18). This indicates that in MG1655 deletion of *cpxR* does not affect growth at low pH.

In addition, a number of genes that were not previously shown to be regulated by the CpxA/R TCS were also identified. Members of the *glc* operon (*glcD*, *glcE* and *glcF*) were shown to be expressed at a significantly higher level in the *cpxR* mutant compared to wildtype suggesting a role for the Cpx pathway in regulating glycolate utilization in HM605. Furthermore, members of the *pgaABCD* operon, encoding proteins required for the production of PGA (Poly-beta-1,6-N-acetyl-d-glucosamine), an EPS involved in biofilm formation (Wang *et al.*, 2004), was also shown to be expressed at a significantly higher level in the *cpxR* mutant. The Cpx TCS has been reported to affect biofilm formation by regulating the production of curli fimbriae in response to osmotic conditions (Prigent-Combaret *et al.*, 2001) and by playing a key role in the regulation of adhesion-induced gene expression (Otto & Silhavy, 2002). This result suggests the involvement of the Cpx pathway in negatively regulating the *pgaABCD* operon. Production of PGA can be discriminated on congo red agar (red-stained cells indicated the presence of the EPS driven by the *pgaABCD* operon) (Ionescu & Belkin, 2009).

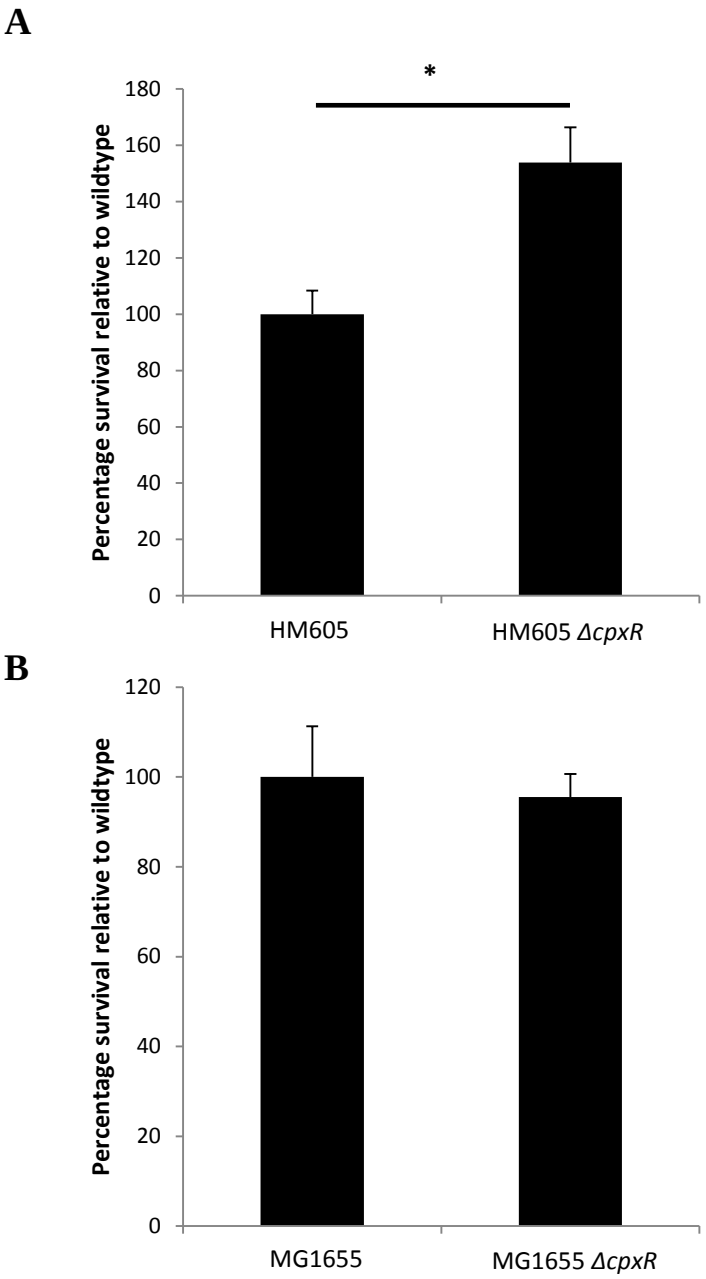


Figure 4.18: Acid challenge. The graph represents the mean results of 3 cultures per strain replicated in triplicate. HM605*ΔcpxR* is more tolerant to low pH than the wildtype. No differences were observed between MG1655 and MG1655*ΔcpxR*. For analysis of the significance of differences in numbers, Student's t-test was used for comparison of two groups of data (∗ P-value ≤ 0.05).

However, no difference in PGA production was observed between wildtype and the *cpxR* mutant when assessed on Congo red agar (data not shown). Studies have reported that Congo red binding does not always correlate with PGA production, despite its widespread use as an indicator of biofilm production (Yoong *et al.*, 2012). Therefore, the possible involvement of the Cpx pathway in negatively regulating the *pgaABCD* operon should not be disregarded.

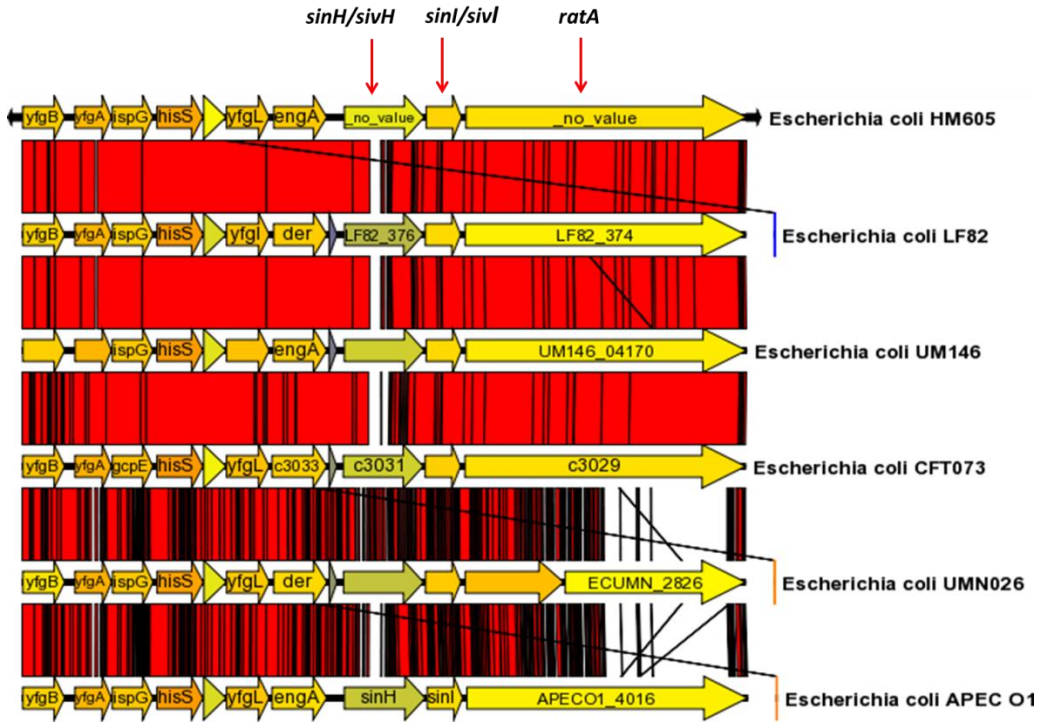
It is important to note that a number of results from the microarray have previously been verified in this study independent of the microarray. For example, from the microarray both *ycfS* and *yebE* are both significantly down-regulated in *ΔcpxR* and previous transcriptional gfp-promoter fusions confirm that the level of expression of both *ycfS* and *yebE* is reduced in *ΔcpxR* compared to wildtype (fig 4.5). Moreover, microarray analysis show that *rpoE* is significantly up-regulated in *ΔcpxR* (1.6 fold) and previous transcriptional gfp-promoter fusions confirm that *rpoE* expression is up-regulated in *ΔcpxR* compared to wildtype. Furthermore, a number of gfp transcriptional fusions were made independently of this study by Dr. Emma Smith, Alimentary Pharmabiotic Centre (APC), University College Cork (personal communication) which verified a number of results from the microarray. For example, from the microarray *pgaA*, *glcD*, *hdeA*, *cysD* and *yagU* were expressed significantly higher in the *ΔcpxR* mutant compared to wildtype. The gfp transcriptional fusions constructed by Dr. Emma Smith confirmed that the level of expression of each of these genes is significantly higher in *ΔcpxR* compared to wildtype. In addition, we previously showed that show that HM605*ΔcpxR* was significantly more tolerant to low pH as it was able to survive better at pH 2.5 compared to wildtype HM605 (see fig 4.18). Together, these results help to support the findings of the microarray.

4.3.9.2 Cpx A/R pathway positively regulates the expression of *sinH* in AIEC HM605

The aim of this transcriptome analysis was to identify invasion factors that were positively regulated by the Cpx A/R TCS i.e down-regulated in *ΔcpxR*. From the analysis, 2 genes were found to be significantly down-regulated, *sivH/sinH* (7.5 fold)

to encode a putative intimin protein/invasion protein. SinH is a member of the family of intimin-like proteins/invasins and it shares a short region of homology with intimin. Intimin is a virulence factor (adhesion) of EPEC and EHEC involved in the attaching and effacing (A/E) phenotype and has recently been reported to be important for the intracellular pathogen *Yersinia pestis* (Seo *et al.*, 2012; Stevens *et al.*, 2006). Analysis of SivH/SinH shows it to be primarily associated with *E. coli* and *Salmonella* spp. showing strong homology with a gene found in sequenced UPEC strains, AIEC strains (including LF82), NMEC strains and *S. enterica*. Moreover, in *S. enterica* serovar Typhimurium, *sivH/sinH* has been shown to contribute to the colonization of Peyer's patches (Kingsley *et al.*, 2003) and it has recently been predicted to be a potential invasion factor in AIEC strain NRG857c (Nash *et al.*, 2010). The genes encoding SinH and SinI are always found together with a 3rd gene called *ratA*, encoding a putative outer membrane protein which was not identified on our array as being regulated by the Cpx pathway (see fig 4.19). The amino acid sequence of SinH in HM605 exhibited strong homology with SinH of other *E. coli* (> 94% identity) primarily the AIEC strain UM146 and *E. coli* APEC O1 (see Sig 4.19). However, gaps in some of the nucleotide sequences of *sinH* suggest possible transfer or deletion events (see fig 4.19 A). It could be hypothesised that such events could lead to differential expression of the *sinH* gene between *E. coli* strains. Ultimately, the presence of *sivH*, *sivI*, and *ratA* in a number of *E. coli* strains suggests a complex evolutionary history of this region.

A



B

Strain	Locus tag for *sinH*	Length (aa)	Score relative to HM605
LF82	LF82_376	725	97.0
UM146	UM146_04160	734	100.0
CFT073	c3031	735	95.0
UMN026	ECUMN_2829	735	94.0
APEC O1	sinH	743	100.0

Figure 4.19: A) The loci of *sinH* and *sinI* in *E. coli*. These genes are associated with *E. coli* and *Salmonella* spp. and are always located near *ratA* and *engA* (*der*) which encodes a GTP –binding protein. Both of which were not shown to be regulated by the Cpx pathway in this study. **B) ClustalW multiple sequence alignment analysis comparing SinH in other *E. coli* to HM605 (734 aa).** Amino acid sequence shows strong homolog with other *E. coli*.

The deduced amino acid sequence of *sivH/sinH* in HM605 exhibited homology in its N-terminal with intimin of EPEC. The amino termini of intimin serve as a membrane-spanning anchor in the bacterial outer membrane. Homology with the N-termini of intimin suggests that SivH may be an integral outer membrane protein. The potential location of this protein suggested a possible involvement with the Cpx envelope stress response and it is possible to hypothesis that alterations in the membrane by deletion of *cpxR* could have a significant effect on the folding of the SivH/SinH protein resulting in the invasion defect observed in *ΔcpxR*. In *S. enterica* serovar Typhimurium, *sivH/sinH* contributes to the colonization of Peyer's patches (Kingsley *et al.*, 2003) and it has recently been predicted, based on in silico analysis, to be a potential invasion factor in AIEC strain NRG857c (Nash *et al.*, 2010). Interestingly, further analyse of the amino acid sequence of *sivH/sinH* in HM605 using SignalP 4.0 (Petersen *et al.*, 2011) identified the presence of an N-terminal signal peptide (residues 1 to 20) and signal peptide cleavage site between positions 19 and 20. The presence of a signal peptide indicates a potential secretion mechanism for SinH. This signal peptide was conserved in the amino acid sequence of *sivH/sinH* in all *E. coli* strains examined (see fig 4.19).

In order to confirm that *sinH* expression is regulated by the CpxA/R TCS a *sinH*-gfp transcriptional fusion was constructed using plasmid pUA66. The plasmid was transformed into wild-type and *ΔcpxR* mutant cells containing the *nlpE* overexpressing plasmid and overnight cultures of the test strains were diluted to OD_{600} =0.05 in 5mls of LB. Cultures were grown until OD_{600} 0.5 and 10µM IPTG was added to induce the expression of NlpE and growth and fluorescence were monitored for several hours. This transcriptional fusion confirmed that *sinH* expression was significantly down-regulated in the *cpxR* mutant (4.2 fold) compared to the wild-type (see fig. 4.20). A *sinI*-gfp transcriptional fusion was also constructed using plasmid pUA66 however no significant GFP expression was detected suggesting that *sinH* and *sinI* may be transcribed as an operon (see Fig. 4.20).

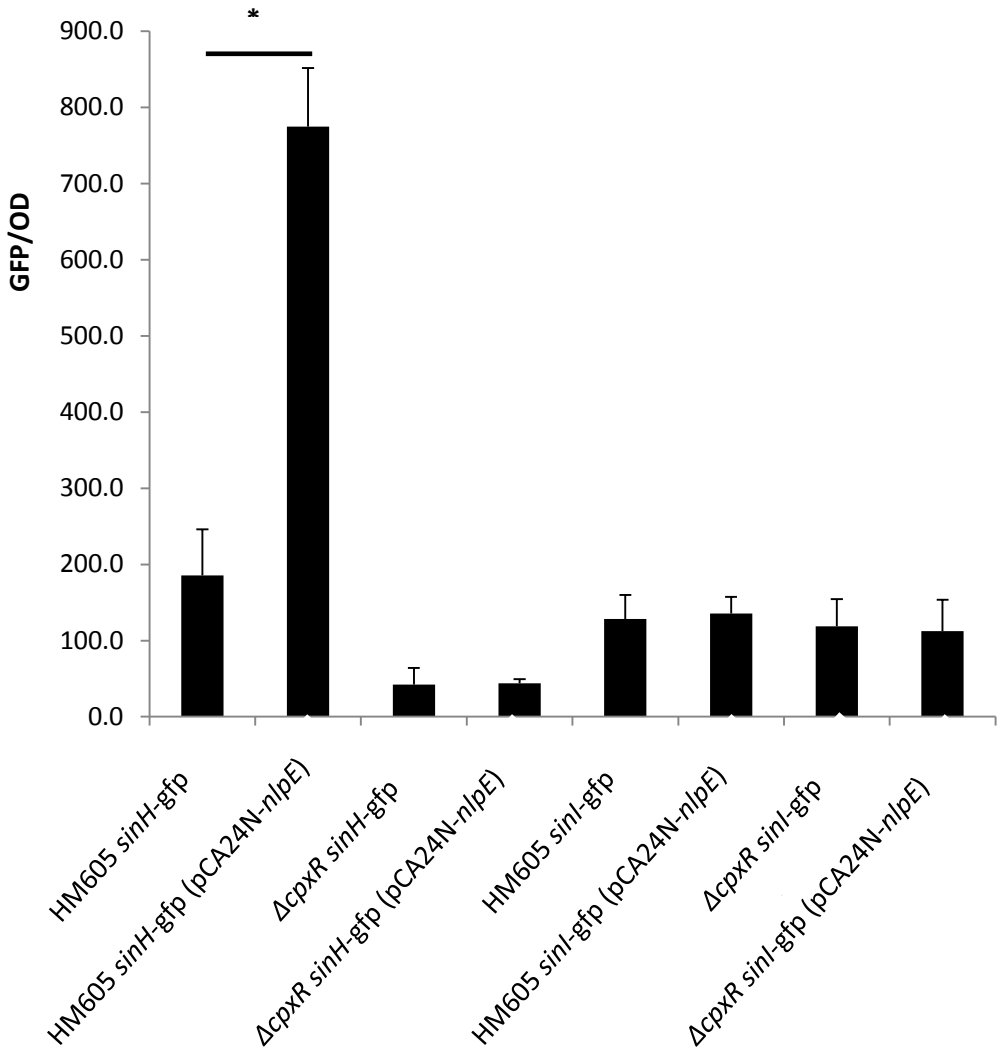


Figure 4.20: Expression of sinH and sinI using gfp reporter fusions. Cells were grown under inducing conditions for 12 hours and OD_{600} and fluorescence was measured after 12 hours. In the wildtype, activation of the wildtype CpxA/R pathway using NlpE results in a significant increase in fluorescence from cells carrying the *sinH*-gfp fusion. Each strain was represented by 3 individual cultures. For analysis of the significance of differences in expression, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

Comparing HM605 *sinH*-gfp + *nlpE* and *ΔcpxR sinH*-gfp + *nlpE* is equivalent to the microarray (except GFP is done at 12 hours, not 30 minutes); nonetheless, it is clear the expression of *sinH* is down-regulated in the *cpxR* mutant. Also, there is a Cpx-dependent increase in *sinH* when NlpE production is increased confirming the positive regulation of *sinH* by the Cpx pathway.

4.3.9.3 Investigating the role of *sinH* in the invasion of HM605*ΔcpxR*

The results of the microarray analysis revealed that the Cpx TCS positively regulates the expression of *sinH*. Unfortunately numerous attempts to make a direct knockout of *sinH* in HM605 failed. Indeed, no directed knockouts have been constructed in HM605 to date. Therefore, in order to try and implicate *sinH* with a role in invasion, it was decided to overexpress *sinH* from a plasmid (pBAD33), under the control of the *araBAD* promoter. Therefore, expression of *sinH* can be induced by the addition of 0.2% (w/v) L-arabinose to the growth medium and will be independent of CpxR. Adhesion and invasion experiments were set up as previously described and it is clear that the overproduction of SinH does not affect the ability of the HM605 bacteria to adhere to C2Bbe1 epithelial cells (see Fig 4.21). However, the overproduction of SinH did restore the invasion defect observed in the *cpxR* mutant (see fig 4.21). These findings indicate that the invasion defect observed in HM605Δ*cpxR* is, at least partially, due to the defect in *sinH* expression observed in the *cpxR* mutant strain. Although a directed knockout of *sinH* in HM605 has not been constructed, these results suggest that SinH is involved in the invasion of epithelial cells by HM605.

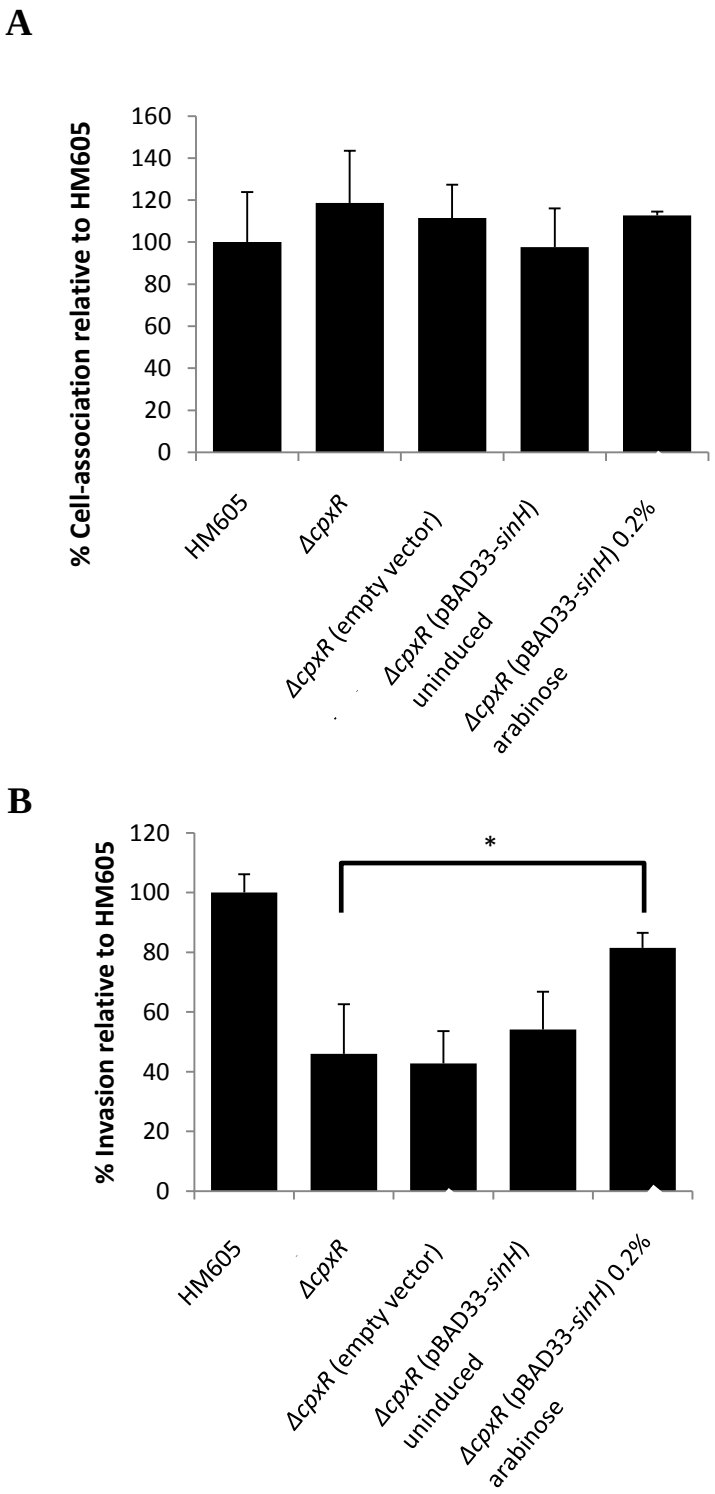


Figure 4.21: Complementation of HM605Δ*cpxR* with cloned *sinH* restores invasion into C2Bbe1 epithelial cells. (A) Adhesion to C2Bbe1 epithelial cells. Cell-associated bacteria were quantified after a 3-h infection period. The results are expressed as levels of cell-associated bacteria (adherent plus intracellular) relative to those obtained for wild-type strain HM605, taken as 100%. (B) Bacterial invasion of C2Bbe1 epithelial cells was determined after gentamicin treatment for an additional 1 h. The results are expressed as levels of intracellular bacteria/associated bacteria relative to those obtained for wild-type strain HM605, taken as 100%. For analysis of the significance of differences in association and invasion into C2Bbe1 epithelial cells, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

4.4 Conclusion

The objective of this chapter was to examine the role of two component signal transduction pathways in the pathogenesis of the AIEC strain HM605. In total 11 systems were tested and initial findings suggested that none of the two component pathways examined were required for AIEC HM605's ability to replicate in J774.A1 macrophages. However, it was observed that the Δ*cpxR* mutant showed an 85% reduction in internalisation after 1 h compared to wildtype (see Fig 4.2). In support of this observation, despite having no effect on adhesion to epithelial cells, a deletion of the *cpxR* gene resulted in a significant ($P \leq 0.05$) decrease in the ability of the HM605 to invade C2Bbe1 epithelial cells (see Fig 4.6). These results suggested a potential role for the Cpx TCS in the interaction of HM605 with eukaryotic cells.

The CpxA/R TCS is an envelope stress response system that mediates the cellular response to envelope protein misfolding (Price & Raivio, 2009). Furthermore, the size of the Cpx regulon (50 genes in 34 operons) and the diversity of the physiological functions associated with the genes controlled by the Cpx TCS (mobility, adherence factors, metabolism, etc.) indicate that this signalling pathway has a complex role in controlling bacterial physiology (Dorel *et al.*, 2006; Price & Raivio, 2009; Raivio, 2005). As previously stated, the CpxA/R TCS has been shown to be involved in the expression of the type IV bundle-forming pilus (BFP) of EPEC (Leuko & Raivio, 2012; Vogt *et al.*, 2010). EPEC strains lacking CpxR exhibit diminished auto-aggregation and adherence to eukaryotic cells, both of which are BFP-mediated (Raivio, 2005; Vogt *et al.*, 2010). The Cpx pathway has also been implicated in the virulence of other pathogenic Gram-negative bacteria. For example, the invasive intestinal pathogens *Shigella flexneri* and *S. enterica* serovar Typhimurium contain CpxP, CpxR, CpxA proteins that are highly conserved (>95% identity) relative to those of *E. coli* (De Wulf *et al.*, 1999; Raivio, 2005). In *S. enterica* serovar Typhimurium gain of function mutations in the *cpxA* gene lead to a reduction and invasion of eukaryotic cells as well as impaired ability to grow in mice (Humphreys *et al.*, 2004). Recently, *Sh. sonnei cpxA* mutants were also shown to affect the post-transcriptional levels of the InvE regulatory protein, a second central regulator of virulence genes, including those encoding the type III secretion system (T3SS) (Mitobe *et al.*, 2005; Raivio, 2005). Cpx pathway activation also restricts levels of the RovA global regulator of gene expression in pathogenic *Yersinia*

pseudotuberculosis (Liu *et al.*, 2011). These examples highlight the important role of the Cpx pathway and suggests that, as in *E coli*, the Cpx pathway may work at both transcriptional and post-transcriptional levels to affect pathogenesis (Raivio, 2005).

Numerous studies have identified flagella and type 1 fimbriae as essential invasion factors in AIEC (Barnich *et al.*, 2003; Eaves-Pyles, 2008). However in contrast, this study has shown that neither of these factors is important for the invasion of epithelial cells by HM605. This provides further evidence for our previously reported observation that AIEC are a phenotypically diverse group (see Chapter 3.3.7). Initial findings from swim agar plates (see Fig 4.7) confirmed that motility was reduced in the HM605 Δ*cpxR*::Km mutant compared to wild-type (see Fig 4.7). Interestingly, previous studies with the *E. coli* K-12 strain W3110 showed that activation of the Cpx pathway severely impaired motility due to the negative regulation of *motAB* (motility) and *cheAW* (chemotaxis) (De Wulf *et al.*, 1999; Price & Raivio, 2009). In contrast, the findings in this study indicate that the Cpx pathway may positively regulate motility in *E. coli*. However there is no evidence in the microarray to suggest a transcriptional effect on motility. Interestingly, analyses of the Cpx regulon in *E. coli* MC4100 showed moderate negative Cpx-mediated regulation of the transcription of *motAB- lux* fusions (Price & Raivio, 2009). A possible explanation for reduced motility in the absence of *cpxR* in these strains could be the result of physical changes to the cell membrane. In the entomopathogenic bacterium *Xenorhabdus nematophila*, a *cpxR* deletion results in reduced motility and lipase activity as well as reduced virulence. This reduction in motility is hypothesised to be caused by alterations in flagellar type III secretion caused by physical changes in the cell membrane (Herbert *et al.*, 2007).

The transcriptome results revealed that a large number of genes were up-(409 genes) or down-(524 genes) regulated in *ΔcpxR*, compared to wildtype, by > 2 fold following the activation of the pathway by NlpE. One interesting observation was that a number of acid resistance genes were significantly up-regulated in *ΔcpxR*, primarily *hdeA, hdeB, hdeD* and *gadE* and this has led to the cpxR mutant being significantly more resistant to acid stresses than the wildtype. In contrast, no difference in acid resistance was observed between MG1655 and MG1655*ΔcpxR*.

This could possibly be an adaptation by some *E. coli* strains to better colonise the gut. Furthermore, the EvgS/A TCS is involved in acid resistance and acid resistance in exponentially growing cells is induced by the overexpression of EvgA (Eguchi *et al.*, 2007). It has been reported that EvgA also directly induces GadE expression (Eguchi *et al.*, 2007; Ma *et al.*, 2004). Subsequently, GadE directly up-regulates *hdeA*, the periplasmic chaperone gene protecting the cell from organic acid stress, and *hdeD*, the putative membrane protein gene participating in acid resistance (Eguchi *et al.*, 2007; Hommais *et al.*, 2004; Ma *et al.*, 2004; Mates *et al.*, 2007). These data indicate a possible link between the CpxA/R pathway and the EvgS/A system in regulating the expression of acid resistance genes.

The most interesting observation was the significant down-regulation of two genes, *sivH/sinH* (7.50 fold down) and *sivI/sinI* (13.35 fold down). The first, *sivH/sinH* encodes a putative intimin protein/invasion protein. Homology with the N-termini of intimin suggested that SivH may be an integral outer membrane protein and the identification of an N-terminal signal peptide and signal peptide cleavage site indicates a possible mechanism of secretion. The potential location of this protein suggests involvement of the Cpx TCS in its regulation. Furthermore, homology with the N-termini of invasin of *Yersinia pseudotuberculosis* suggests a possible role for this protein in invasion as the virulence factor *invasin* allows efficient entry of *Y. pseudotuberculosis* into mammalian cells (Dersch & Isberg, 1999). Interestingly, a mutation in *sivH* reduces colonization of Peyer's patches but does not affect the ability of *S. enterica* serotype Typhimurium to colonize cecum or faeces of mice (Kingsley *et al.*, 2003). Moreover, in *S. enterica* serotype paratyphi A, it has been suggested that the conserved pseudogene *sivH* may be involved in the convergent adaptation of *Salmonella* to its host (Holt *et al.*, 2009).

The findings presented in this chapter suggest that the Cpx TCS is required for the invasion of AIEC HM605 into C2Bbe1 epithelial cells. Furthermore, this invasion defect is independent of known AIEC invasion factors of AEIC LF82 (flagella and type 1 fimbriae). Transcriptome results revealed that the CpxA/R TCS positively regulates the expression of a putative intimin/invasion protein SinH (SivH) and overproduction of SinH restored the invasion defect in the *cpxR* mutant to levels

similar to wildtype. The presence of SinH in a number of pathogenic *E. coli*, suggest it may have an important role in the virulence of other *E. coli*. The results suggest that role of the CpxA/R TCS in AIEC HM605 is to optimise the expression of the protein SinH to result in successful invasion into C2Bbe1 epithelial cells.

Chapter 5.0: Oxidative stress and the intramacrophage survival of adherent invasive *Escherichia coli* strain HM605

5.1 Introduction

Oxidative stress, through the production of reactive oxygen species (ROS), is an unavoidable consequence of aerobic metabolism (Chiang & Schellhorn, 2012). Oxidative stress arises when the concentration of active oxygen species increases to a level that exceeds the cell's capacity to remove these harmful molecules (Cabiscol *et al.*, 2000). Therefore, the ability to maintain intracellular concentrations of ROS within safe limits is essential for all aerobic life forms (Dubbs & Mongkolsuk, 2012). Genetic responses to oxidative stress occur in bacteria, yeast, and mammalian cell lines (Cabiscol *et al.*, 2000). ROS, including superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2) are known to cause damage to DNA, RNA, protein and lipids (Mukhopadhyay & Schellhorn, 1997). For example, free radicals can directly attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation which alters membrane properties and disrupts membrane-bound proteins (Cabiscol *et al.*, 2000). In addition to being an unavoidable consequence of respiration, bacteria can encounter toxic levels of ROS produced by macrophages, neutrophils and other phagocytic cells during phagocytosis (Cabiscol *et al.*, 2000; Mukhopadhyay & Schellhorn, 1997).

Bacteria and other organisms possess several enzymes that prevent oxidative damage and repair DNA lesions resulting from oxidative damage (Mukhopadhyay & Schellhorn, 1997). For example, *E. coli* cells are equipped with two superoxide dismutases (SODs), a manganese containing SOD (Mn-SOD, encoded by *sodA*) and an iron containing SOD (Fe-SOD, encoded by *sodB*) (see Fig. 5.1) (Cabiscol *et al.*, 2000; Farr & Kogoma, 1991). The expression of *sodB* is modulated by intracellular iron levels and *sodA* (encoding the predominant enzyme during aerobic growth) expression is transcriptionally regulated by at least six control systems (Compan & Touati, 1993; Niederhoffer *et al.*, 1990).

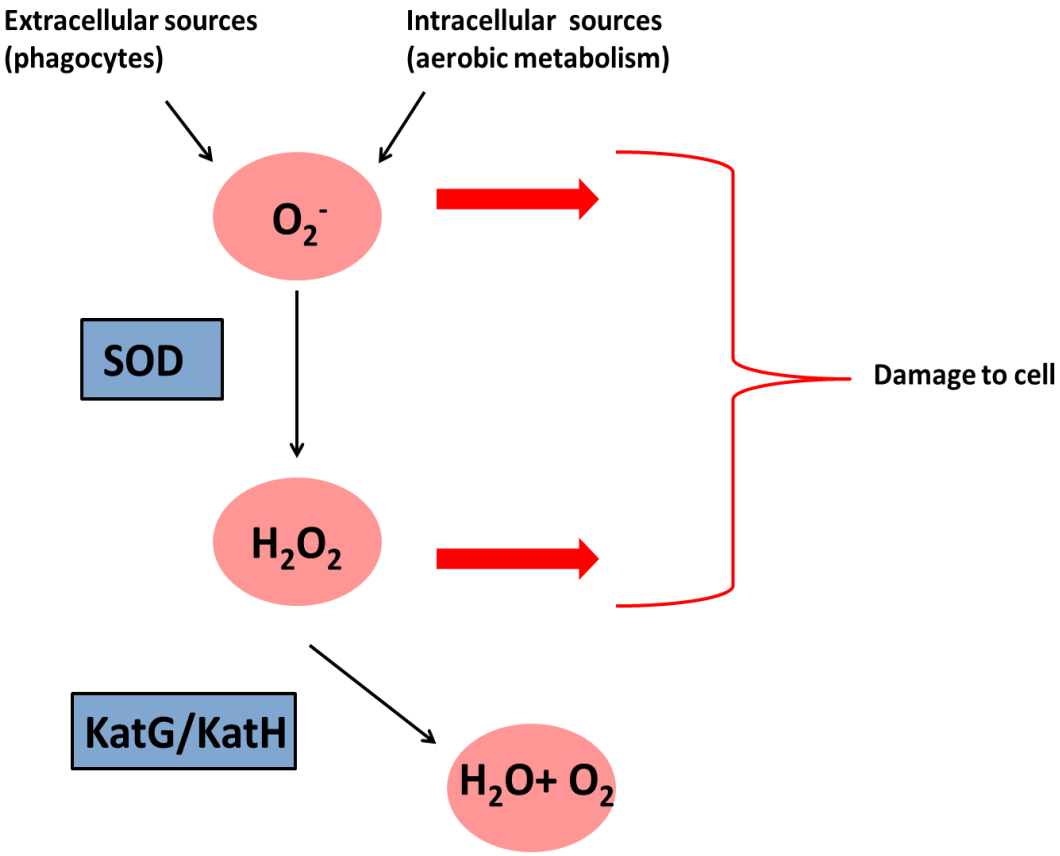


Figure 5.1: Bacterial defence against oxidative damage. *E. coli* and other organisms use superoxide dismutase, catalases, and peroxidases to scavenge O_2^- and H_2O_2 preventing damage to the cell. O_2^- does not react directly with polypeptides but it has become clear that O_2^- can disable the TCA cycle and branched-chain biosynthetic pathways by destroying the iron-sulphur clusters of critical enzymes causing cell damage. H_2O_2 reacts with the intracellular pool of unincorporated ferrous iron and thereby generates hydroxyl radicals which react with virtually all biomolecules such as DNA (modified from Mertens.*et al* 2005).

SODs function by converting the superoxide anion (O_2^-) into H_2O_2 and O_2 (Cabiscol *et al.*, 2000; Farr & Kogoma, 1991; Mertens *et al.*, 2005). Furthermore, *E coli* possess two catalases: hydroperoxidase I (HPI) and hydroperoxidase II (HPII) that convert H_2O_2 to H_2O and O_2 (see Fig. 5.1) (Cabiscol *et al.*, 2000).

5.1.1 Antioxidant defense systems of *Escherichia coli*

E. coli cells possess a specific defense against peroxides, mediated by the transcriptional activator OxyR, and another against superoxide, controlled by the SoxR/S regulon (Cabiscol *et al.*, 2000). OxyR and SoxR undergo conformational changes when oxidized in the presence of H_2O_2 and O_2^-, respectively, and subsequently control the expression of cognate genes (Chiang & Schellhorn, 2012). In response to an increase in H_2O_2 and other organic peroxides the cellular concentrations of at least 30 proteins become elevated over basal levels. The induction of 9 of the 30 proteins is under the positive control of OxyR, a member of the LysR family of transcriptional regulators (Christman *et al.*, 1985; Christman *et al.*, 1989; Mukhopadhyay & Schellhorn, 1997). The OxyR protein is a dimer that acts both as a sensor of oxidative stress and as the transcriptional activator for the regulon (Seaver & Imlay, 2001b). Furthermore, OxyR is found in a large number of bacteria and has been shown to be vital for survival after exposure to ROS, including H_2O_2. OxyR is reversibly activated by the formation of an intramolecular disulfide bond between the 2 subunits of the OxyR, resulting from the altered redox state of the cell (Cabiscol *et al.*, 2000). The resulting conformational change allows the protein to bind to its target sequences and activate expression of proteins in the regulon (Zheng *et al.*, 1998). Using the formation and reduction of a disulfide bond as an "on-off" switch allows for a rapid response to changes in the oxidative conditions within the cell (Cabiscol *et al.*, 2000). OxyR controls the induction of, amongst others, *katG* (encoding HPI catalase), *ahpCF* (encoding NADPH-alkyl hydroperoxidase reductase), *gorA* (glutathione reductase) and *dps* (a protective DNA-binding protein) (Cabiscol *et al.*, 2000; Farr & Kogoma, 1991; Mukhopadhyay & Schellhorn, 1997; Seaver & Imlay, 2001b). The SoxR/S regulon is controlled by the SoxR sensor protein and the SoxS transcription factor that activates genes in the regulon (Storz & Imlay, 1999). The SoxR/S regulon contains at least ten genes, including those encoding the Mn-SOD which removes the superoxide anion,

endonuclease IV, glucose-6-P dehydrogenase, fumarase C, aconitase A, ferredoxin reductase and *micF* RNA (Cabiscol *et al.*, 2000; Liochev & Fridovich, 1992; Pomposiello *et al.*, 2003; Seaver & Imlay, 2001a; Seaver & Imlay, 2004; Storz & Imlay, 1999). SOD synthesis is positively regulated by the SoxR/S regulon, which convert O_2^- to H_2O_2 and O_2. As H_2O_2 is harmful it needs to be detoxified and this is achieved via enzymes encoded by genes under the control of OxyR (Storz & Imlay, 1999). Evidence from a number of studies shows interplay between antioxidant defense systems and DNA repair mechanisms (Hassett & Cohen, 1989; Lombard *et al.*, 2005; Mukhopadhyay & Schellhorn, 1997; Suvarnapunya *et al.*, 2003). For example, OxyR is involved in the up-regulation of *uvrD*, which encodes a DNA helicase II involved in nucleotide excision repair (NER) and mismatch repair (MMR) (Mukhopadhyay & Schellhorn, 1997). Such studies highlight a relationship between the defense against oxidative stress and the maintenance of the cells genomic integrity.

5.1.2 Oxidative damage to bacteria by macrophages

Macrophages represent a powerful defense system against invading microorganisms and their antimicrobial activity is mediated, in large part, by an array of reactive molecules that have bactericidal activity. These include ROS such as H_2O_2, and reactive nitrogen species (RNS), such as nitric oxide (Suvarnapunya *et al.*, 2003). When macrophages kill micro-organisms, they ingest them into phagosomes and bombard them with a burst of ROS (Winterbourn & Kettle, 2012). Within host cells, ROS are generated by multiple mechanisms including the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), myeloperoxidase, lipoxygenases, and cellular respiration (Llewellyn *et al.*, 2011). The phagocyte NADPH oxidase (phox), a multiprotein complex, exists in the dissociated state in resting cells (Robinson, 2008). Upon engulfment, the unique membrane bound NADPH-dependent oxidase complex is activated and transfers electrons from NADPH in the cytoplasm to oxygen in the phagosomal lumen yielding superoxide. Superoxide is unstable and rapidly reduced (spontaneously or enzymatically by SOD) to H_2O_2 in a pH dependent fashion. The formation of H_2O_2 is favoured at the acid pH achieved in the phagocytic vacuole (Hassett & Cohen, 1989; Splettstoesser & Schuff-Werner, 2002; Winterbourn & Kettle, 2012). Physiological stimuli capable of activating the

NADPH oxidase include particles such as bacteria and yeast, certain molecules that induce chemotaxis in these cells, some bioactive lipids, and antibodies (Robinson, 2008). Neutrophils (but not macrophages) contain high concentrations of myeloperoxidase (MPO), an enzyme that catalyses the conversion of H_2O_2 to hypochlorous acid (HOCL), provided adequate chloride is available. Studies with specific probes have shown that HOCl is produced in the phagosome and reacts with ingested bacteria (Hassett & Cohen, 1989; Winterbourn & Kettle, 2012). In addition, nitric oxide (NO^-) synthesis, via the inducible nitric oxide synthase (iNOS), also occurs (Suvarnapunya *et al.*, 2003). NO^- acts as a destructive molecule destroying cancer cells, parasites, and intracellular pathogens and is produced in large amounts by activated murine macrophages (Splettstoesser & Schuff-Werner, 2002). NO^- production is independent from ROS but the interaction of NO^- with ROS leads to the formation of peroxynitrite ($ONOO^-$), a potent oxidant (Splettstoesser & Schuff-Werner, 2002). The consequence of these different activities is that phagocytosed bacteria are killed by oxidative damage that targets a range of molecules, including DNA, RNA, protein and lipids (Suvarnapunya *et al.*, 2003).

Even within this potentially hostile environment, a number of bacteria persist and survive inside macrophages. *S. enterica* serovar Typhimurium possess a type 3 secretion system encoded by the *Salmonella* pathogenicity island 2 (SPI2) which allows *Salmonella* to limit its exposure to macrophage oxidants by disrupting the targeting of phox to the *Salmonella*-containing vacuole (SCV) (Suvarnapunya *et al.*, 2003; Vazquez-Torres *et al.*, 2000). Repair of oxidative DNA damage has also been implicated; as disruption of *recA* attenuates *Salmonella* virulence and base excision mediated repair of DNA damage resulting from ROS has been implicated in the establishment of *Salmonella* infection (Buchmeier *et al.*, 1993; Suvarnapunya *et al.*, 2003). The intracellular pathogen *Francisella tularensis* employs numerous strategies to resist oxidative stress including limiting the activation of the NADPH oxidase and using multiple enzyme systems to detoxify reactive oxygen compounds (Llewellyn *et al.*, 2011). Furthermore, deletion of the *htrA* stress gene in strain AIEC LF82 induces increased sensitivity of the mutant to oxidative stress caused by H_2O_2 (Bringer *et al.*, 2005). Microbial pathogens have adapted to escape one or more host defences and in the context of bacteria such as AIEC that induce inflammation,

response to oxidative stress can be viewed as an important feature of such adaptation (Hassett & Cohen, 1989).

5.2 Objectives

During phagocytosis, macrophages generate ROS which are involved in antibacterial activity. However, AIEC are able to replicate and survive within macrophages suggesting that AIEC may have adapted to resist killing by ROS. Therefore the aim of this research was to investigate whether we could identify H_2O_2 (oxidative stress) response genes involved in the ability of AIEC HM605 to resist killing by macrophages and to replicate within phagocytic cells.

5.3 Results

5.3.1 The minimum inhibitory concentration (MIC) of hydrogen peroxide on AIEC HM605

In order to facilitate a relatively rapid screening method to identify H_2O_2 sensitive mutants of HM605, the minimum inhibitory concentration (MIC) of H_2O_2 on wildtype HM605 was determined. The MIC of H_2O_2 was determined by dilution of overnight cultures into LB supplemented with H_2O_2 in a 96 well plate. Overnight cultures were grown in LB broth and adjusted to an OD_{600} of 0.5 in 150µl of LB broth. Using a 96-well pin replicator, diluted bacteria were transferred to a new 96 well plate containing LB broth with various concentrations of H_2O_2 ranging from 0.3mM to 0.8mM (increasing in intervals of 0.05mM). Control wells contained LB broth with no H_2O_2 (positive control). As H_2O_2 degrades in the presence of light plates were incubated in the dark at 37°C for 24 hours (Feuerstein *et al.,* 2006). OD_{600} readings were taken to determine the MIC. The MIC is reported as the lowest concentration of test material which results in 100% inhibition of growth of the test organism. The data indicated that HM605 could grow in concentrations of H_2O_2 ranging from 0.3mM to 0.65mM (see fig 5.2). However, complete inhibition of growth of HM605 was observed at concentrations above 0.65mM (see fig 5.2). Therefore, from the results obtained, an MIC of 0.6mM H_2O_2 was sufficient to enable its use in a rapid screening method to identify H_2O_2 sensitive mutants of HM605. The MIC of H_2O_2 identified was 0.7mM for HM605 therefore we choose 0.6mM for the screen so we could identify mutants that had even small increases in sensitivity to H_2O_2.

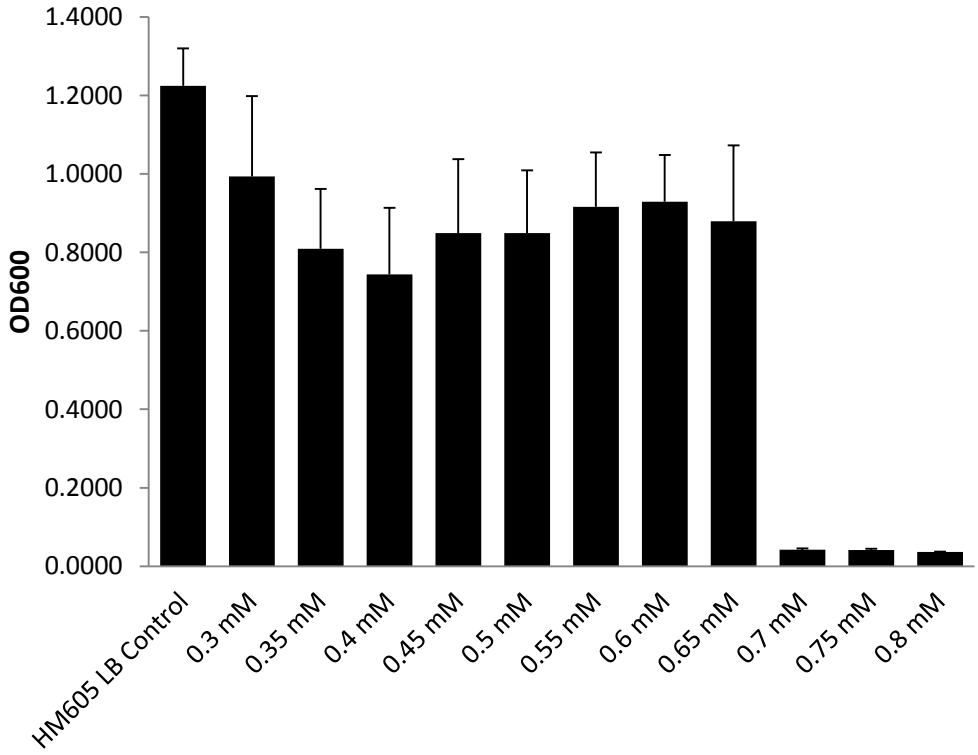


Figure 5.2: The minimum inhibitory concentration (MIC) of hydrogen peroxide on HM605. Overnight cultures were grown in LB broth adjusted to 0.5 OD_{600} in 150µl of LB broth with various concentrations of H_2O_2 ranging from 0.3mM to 0.8mM. The graph represents the average OD_{600} readings from 3 individual cultures. Error bars represent the standard deviation (SD). For analysis of the significance of differences in numbers, Student's t-test was used for comparison of two groups of data.

5.3.2 HM605 Tn5 transposon mutant library screen

In order to identify mutants with increased sensitivity to H_2O_2 5,568 mutants were screened for H_2O_2 sensitivity (see section 2.12). The primary screen resulted in the identification of 12 (0.21% hit rate) H_2O_2 sensitive mutants and these mutants were re-screened to confirm their reduced growth. The secondary screen resulted in the identification of 3 mutants (out of the original 12) showing a complete, and reproducible, reduction of growth in the presence of 0.6mM H_2O_2 (see fig 5.3).

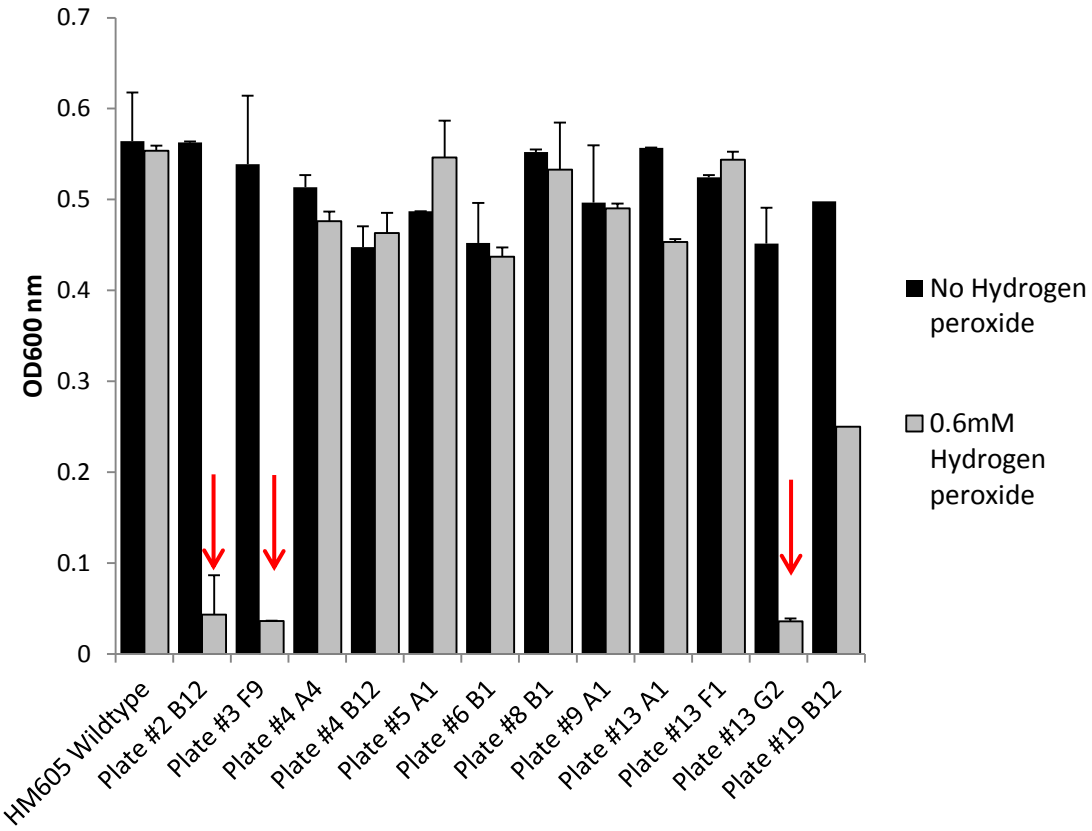


Figure 5.3: Secondary screen to identify hydrogen peroxide sensitive mutants. 3 mutants were identified that exhibited a reproducible H_2O_2 sensitive phenotype. The arrow identifies the mutants showing reduced growth in 0.6mM H_2O_2. The graph represents the average OD_{600} readings from 3 individual cultures. Error bars represent the standard deviation (SD). For analysis of the significance of differences in numbers, Student's t-test was used for comparison of two groups of data (Red arrow represents a P-value ≤ 0.05).

In addition to growth in a 96 well plate, the 3 mutants identified in the secondary screen were examined for their sensitivity to H_2O_2 by an agar overlay diffusion assay (Bertrand *et al.*, 2010). The results from the agar overlay diffusion method (see fig 5.4) confirmed that the 3 mutants identified from the secondary screen showed a reproducible increase in H_2O_2 sensitivity. The halo size in millimetre (mm) of mutants #2:B12 (41.4mm ± 0.7), #3:F9 (39.5mm ± 0.7) and #13:G2 (41.0mm ± 1.4) were significantly increased ($P \leq 0.05$) compared to wildtype HM605 (32.0 mm ± 2.8) (see fig 5.4). Furthermore, the halo size of MG1655 (40.0 mm ± 1.1) was also significantly increased ($P \leq 0.05$) compared to wildtype HM605 indicating that HM605 is more resistant to oxidative stress than MG1655 (see fig 5.4).

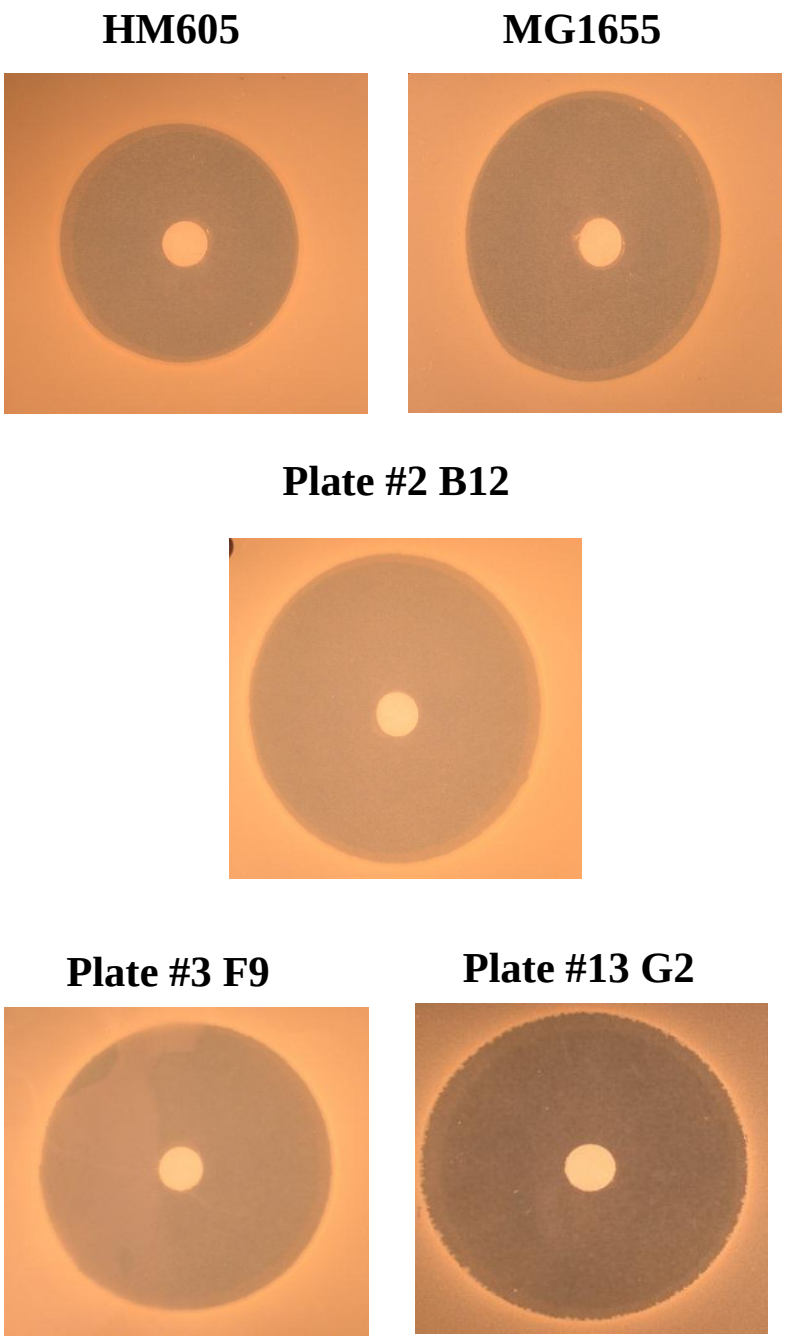


Figure 5.4: Sensitivity to hydrogen peroxide by agar diffusion. Overnight cultures grown in LB broth were adjusted to an OD_{600} = 0.5.and 100µl of each culture was mixed with 4 ml of LB soft agar (0.5% (w/v) agar) and poured onto a LB agar plates. Sterile blank filter paper disks were placed on the surfaces of the solidified soft agar and 10µl of H_2O_2 (30% (v/v)) was spotted onto the disks. The plates were then incubated overnight at 37°C and the diameters of inhibition zones were measured. Shown is the result of a typical experiment. Experiments were carried out in triplicate.

5.3.2.1 Identifying the Tn5 motility minus mutants

Genomic DNA was extracted from all 3 of the H_2O_2-sensitive mutants confirmed as a result of the mutant library screen and the site of insertion was identified using arbritarily-primed PCR (as described previously).

Table 5.1: The insertion site of the Tn5 for each mutant and the identification of the disrupted gene

Mutant ID	Tn5 insertion	Gene disrupted
#2:B12	2002737 bp	*katG*
#3:F9	3721329 bp	*hemF*
#13:G2	2159125 bp	*uvrD*

Mutant #2:B12 was found to have a Tn5 insertion into codon 722 out of 740 for *katG* which encodes the bifunctional HPI hydroperoxidase enzyme, KatG (catalase). The expression of *katG* is known to be regulated by OxyR in a H_2O_2 dependent manner (Mukhopadhyay & Schellhorn, 1997). During oxidative stress *katG* is upregulated resulting in increased production of the hydroperoxidase enzyme which uses a two-electron transfer in the dismutation of H_2O_2 to O_2 and H_2O (Loewen *et al.*, 1985; Mukhopadhyay & Schellhorn, 1997).

The Tn5 insertion mutant #3:F9 was found to have a Tn5 insertion into codon 107 out of 897 for the gene *hemF* which encodes the aerobically produced enzyme known as corpophyrinogen III oxidase. Corpophyrinogen III oxidase catalyses the decarboxylation of coproporphyrinogen III to protoporphyrinogen IX (Troup *et al.*, 1994). This enzyme is required for the production of protoheme IX (Mukhopadhyay & Schellhorn, 1997) which is required for the activity of both HPI hydroperoxidase (encoded by *katG*) and HPII hydroperoxidase (encoded by *katH*) (Loewen *et al.*, 1993). When cultures are exposed to H_2O_2 in exponential phase, there is a rapid increase in *katG* (structural gene for HPI hydroperoxidase) transcription (Morgan *et*

al., 1986; Mukhopadhyay & Schellhorn, 1997). However, to form a functional HPI hydroperoxidase to deal with the challenge, the cell needs to synthesize an adequate amount of protoheme IX. Thus, the *hemF* gene product is required for functional HPI catalase. (Mukhopadhyay & Schellhorn, 1997). *hemF* is reported to be a member of OxyR regulon and a deficiency is reported to render the cell vulnerable to H_2O_2 challenge (Mukhopadhyay & Schellhorn, 1997).

Finally, the insertion site of the Tn5 mutant #13:G2 was found to be in codon 107 out of 721 for *uvrD*, whose product is a DNA helicase II enzyme that is involved in nucleotide excision repair (NER) and also mismatch repair (MM) of DNA. NER is an ubiquitous DNA repair pathway that is found in the sequenced genomes from almost every kingdom, genus, and species that has been examined. The core set of genes involved in the *E. coli* NER system are *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd* (Van Houten *et al.*, 2005). The UvrA/B complex recognizes and binds to the distortion in the DNA duplex caused by DNA damage. UvrB recruits UvrC to the lesion, where it acts as a single-stranded DNA endonuclease cleaving the DNA 5′ and 3′ from the lesion. UvrD (helicase II) then removes the single stranded segment carrying the lesion. DNA polymerase I resynthesizes the DNA copying the undamaged template, and ligase completes the repair (Van Houten *et al.*, 2005). Interestingly, *uvrD* has been identified as a H_2O_2 sensitive mutant in a previously published study identifying H_2O_2 sensitive mutants in *E. coli* (Mukhopadhyay & Schellhorn, 1997).

As 2 of the mutants (*katG* and *hemF*) are involved in the formation of the catalase enzyme these mutants should exhibit reduced bubbling in the presence of H_2O_2 as they should be unable produce a functional catalase enzyme. All 3 H_2O_2 sensitive mutants were tested for catalase activity by flooding the cells with 30% (v/v) H_2O_2 and the results (see fig 5.5) show that all 3 mutants exhibited reduced bubbling in the presence of H_2O_2 compared to wild-type. This indicates that there is reduced catalase activity in all 3 mutants. The reduction in bubbling in the *uvrD* mutant suggests a possible link with OxyR highlighting the previously reported observation that OxyR is involved in the up-regulation of *uvrD* (Modrich, 1994; Mukhopadhyay & Schellhorn, 1997)

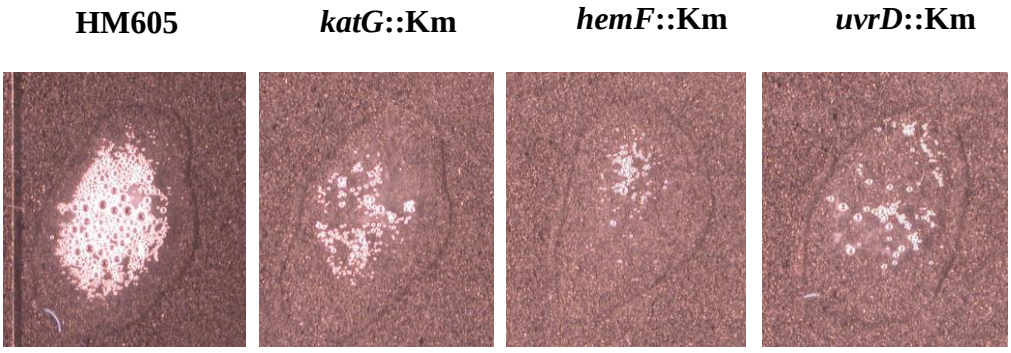


Figure 5.5: Catalase activity of HM605 and hydrogen peroxide sensitive mutants. The H_2O_2 sensitive mutants were tested for catalase activity by flooding colonises of test strains with 30% (v/v) H_2O_2. All mutants tested exhibited reduced bubbling in the presence of H_2O_2, suggesting reduced catalase activity Shown is the result of a typical experiment. Experiments were carried out in triplicate.

5.3.3 The *uvrD* gene is required for survival in the J774 macrophage

In order to determine whether any of these H_2O_2 sensitive mutants are required for HM605 survival in macrophage, the mutants were examined for their ability to survive and replicate inside J774.A1 macrophage. No significant difference in the numbers of intracellular bacteria was observed at either 1h or 6h post-gentamicin between the wild-type strain and the *katG* and *hemF* mutants. This indicates that both of these genes are not required for the survival and replication of HM605. However, the intracellular numbers of the *uvrD* mutant were significantly reduced, at 1h and 6h post-gentamicin, compared to wildtype. Already at 1h post-gentamicin (which corresponds to 3 hours after the bacteria have been added to the macrophage), the number of intracellular HM605 *uvrD*::Km was 19% compared to wild-type and at 6h hours post-gentamicin, the number of intracellular bacteria was 14% compared to HM605. As previously stated, the product of *uvrD* is a DNA helicase II involved in nucleotide excision repair (NER) and also mismatch repair (MM) of DNA. This results suggest that *uvrD* is required for the intracellular survival of AIEC HM605.

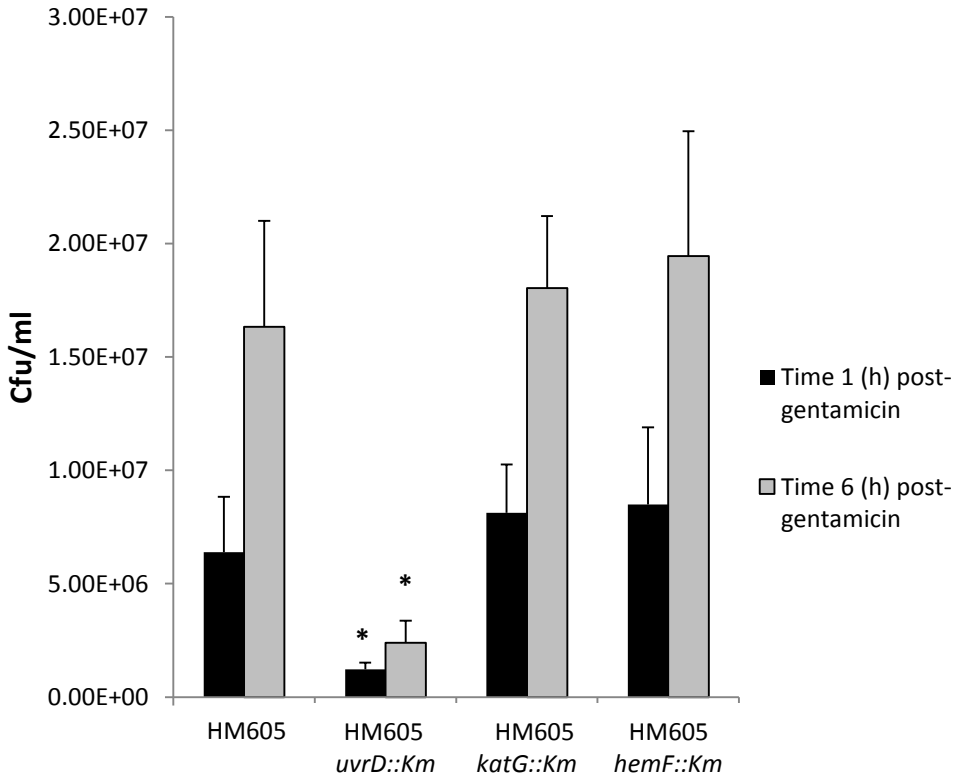


Figure 5.6: Phenotype of H_2O_2 sensitive mutants in J774.A1 macrophage. No significant difference in the numbers of intracellular bacteria was observed at 1h and 6hrs post-gentamicin between the wildtype strain and the *katG* and *hemF* mutants. However, deletion of *uvrD* significantly reduces the intracellular bacterial numbers at 1h and 6hrs post-gentamicin compared to wildtype HM605. Results are from triplicate determinations. For analysis of the significance of differences in uptake and survival within J774.A1 macrophage, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

Macrophages produce ROS and NOS which can damage DNA, amongst other targets (Curti *et al.*, 2007). Therefore, DNA repair systems serve an important housekeeping role in maintaining genomic integrity and may be especially critical in the context of bacterial pathogenesis (Suvarnapunya & Stein, 2005). Moreover, the role of DNA repair systems in the pathogenesis of *S. enterica* serovar Typhimurium and *Helicobacter pylori* has been previously reported (O'Rourke *et al.*, 2003; Suvarnapunya & Stein, 2005). The critical mechanism of both *S. enterica* serovar Typhimurium and *H. pylori* persistence in macrophages is the specific repair of DNA bases oxidized by macrophage oxidants (O'Rourke *et al.*, 2003; Suvarnapunya & Stein, 2005). Interestingly, the intracellular bacterial numbers at 1h and 6h post-gentamicin of the uvrD mutant were significantly reduced compared to HM605. However, transcomplementation of the HM605 *uvrD::Km* mutant with a cloned *uvrD* gene (plasmid pCA24N) restored the intracellular numbers to a level similar to that displayed by the wild-type strain (see fig 5.7).

Therefore the absence of *uvrD* significantly reduced the ability of HM605 to resist prolonged exposure to DNA damaging conditions.

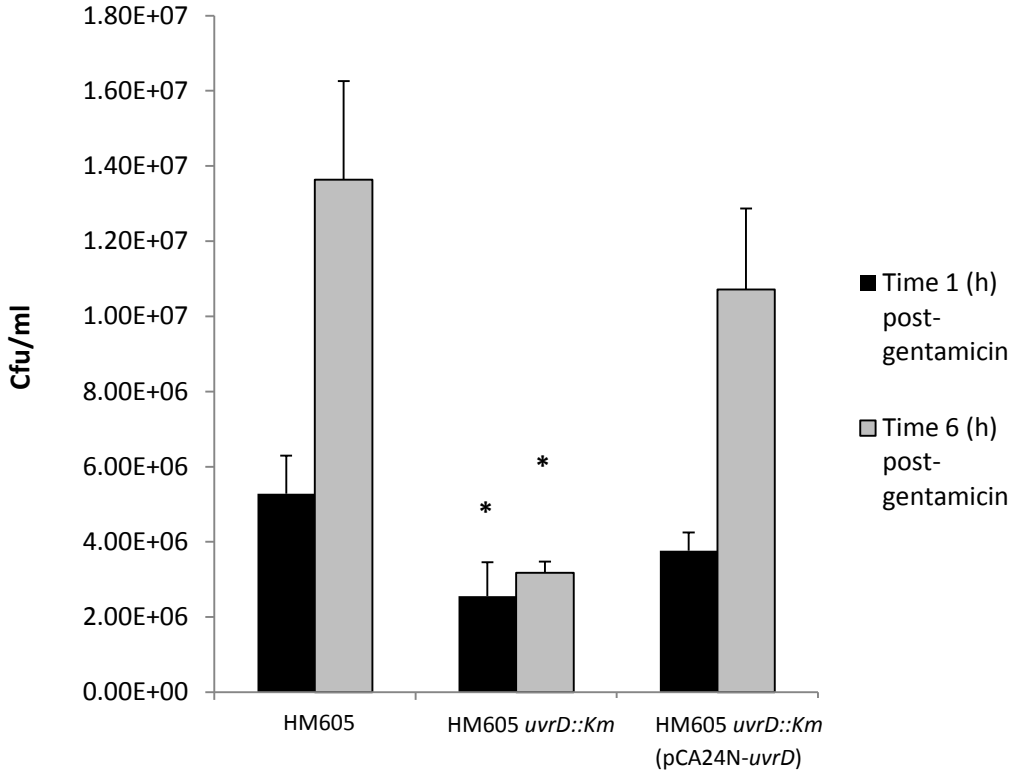


Figure 5.7: Intracellular survival of *uvrD::Km* in J774.A1 macrophage. Deletion of *uvrD* significantly reduces the intracellular bacterial numbers at 1h and 6h post-gentamicin compared to wildtype HM605. Transcomplementation of the *uvrD*::Km mutant with a cloned *uvrD* gene restored the intracellular numbers to a level similar to that displayed by the wild-type strain. Results are from triplicate determinations. For analysis of the significance of differences in uptake and survival within J774.A1 macrophage, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

As previously shown in the agar overlay diffusion method (see fig 5.4), the *uvrD*::Km mutant was significantly more sensitive to H_2O_2 compared to wildtype HM605. Transcomplementation of the HM605 *uvrD*::Km mutant with a cloned *uvrD* gene (plasmid pCA24N) restored the halo size produced by H_2O_2 to a level similar to that displayed by the wild-type strain (see fig 5.8). The halo size in millimetre (mm) of the *uvrD* mutant (39.5mm ± 0.57) were significantly increased ($P \leq 0.05$) compared to wildtype HM605 (33.0 mm ± 0.81) (see Fig 5.8). The expression of *uvrD in trans* was sufficient to restore this phenotype (halo size=35.0mm ± 0.57). This result confirms that *uvrD* is required for the HM605 response to ROS but the sensitivity to H_2O_2 is not the reason why this mutant is unable to replicate in macrophages.

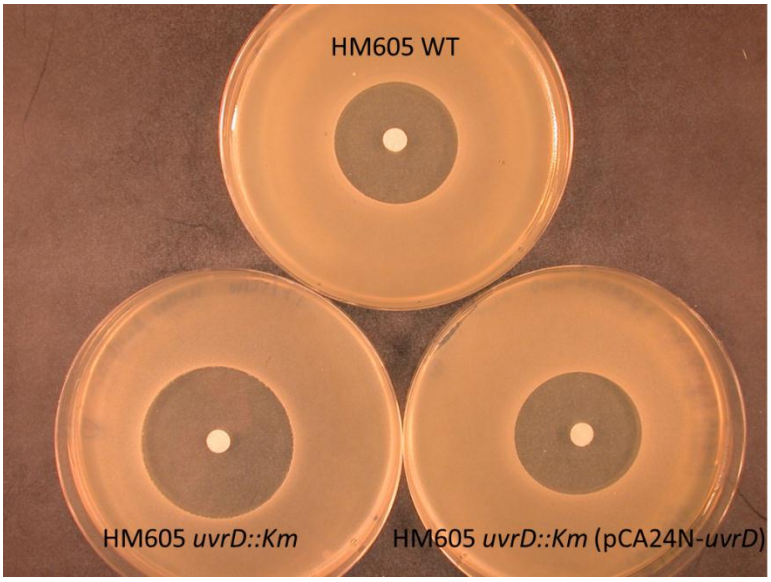


Figure 5.8: Decreased oxidative stress tolerance of the *uvrD*::Km mutant. Transcomplementation of the *uvrD*::Km mutant with a cloned *uvrD* gene restored the halo size produced by H_2O_2 to a level similar to that displayed by the wild-type strain. Shown is the result of a typical experiment. Experiments were carried out in triplicate.

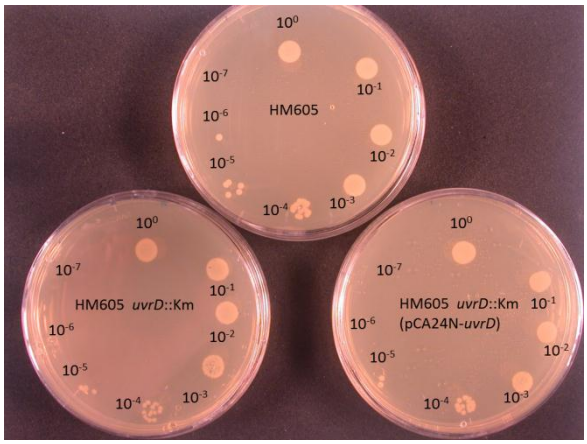
5.3.4 Deletion of *uvrD* affects survival after exposure to nitrosative and oxidative stresses

As previously mentioned, the distinguishing feature of AIEC is their ability to survive and replicate inside macrophages (Bringer *et al.*, 2005). In contrast to other intracellular pathogens, which evade phagocytosis by either escaping the endosome or preventing its fusion with the lysosomal compartment, AIEC replicate within the acidic and harsh vacuolar environment of mature phagolysosomes (Bringer *et al.*, 2006; Simonsen *et al.*, 2011). As the reduced intracellular numbers of the *uvrD* mutant might reflect a low tolerance to conditions characteristic of the mature phagolysosome (e.g. acidic pH, ROS and NOS) we tested the ability of the *uvrD* mutant to grow on agar plates containing various chemical stressors. We already established that the *uvrD* mutant is sensitive to H_2O_2 (see fig 5.8) therefore we also tested acidic pH and NOS. The results of the stress tests are summarised in Figure 5.9.

Resistance to acid was assessed by buffering the LB agar plates with the addition of 100 mM MES pH 5.0 (Simonsen *et al.*, 2011). Bacterial cultures were grown at 37°C in LB medium to an OD_{600}= 0.1 and 5 µl aliquots were spotted onto LB agar plates containing 100 mM MES pH 5.0. Plates were incubated overnight at 37°C. As shown in Figure 5.9, the growth of HM605 *uvrD*::Km was not impaired on LB agar plates adjusted to pH 5.0.

Sensitivity to NOS was assessed by the addition of acidified 1mM sodium nitrite ($NaNO_2$). Under acidic conditions, $NaNO_2$ generates nitrous acid, which decomposes to generate a range of NOS (Kurthkoti *et al.*, 2008). Bacterial cultures were grown at 37°C in LB medium to an OD_{600}= 0.1 and 5 µl aliquots were spotted onto LB agar plates containing 100 mM MES pH 5.0 and 1mM $NaNO_2$. Plates were incubated overnight at 37°C. Inclusion of 1mM acidified $NaNO_2$ severely impeded growth of the *uvrD* mutant while leaving the wild type unaffected (see Fig 5.9). Transcomplementation of the HM605 *uvrD*::Km mutant with a cloned *uvrD* gene (plasmid pCA24N) restored bacterial growth to a level similar to that displayed by the wild-type strain (see fig 5.9). Therefore, the deletion of *uvrD* gene clearly increases HM605 sensitivity to NOS.

A) LB



B) LB + 100 mM MES pH 5.0

C) LB + 100 mM MES pH 5.0 + 1mM $NaNO_2$

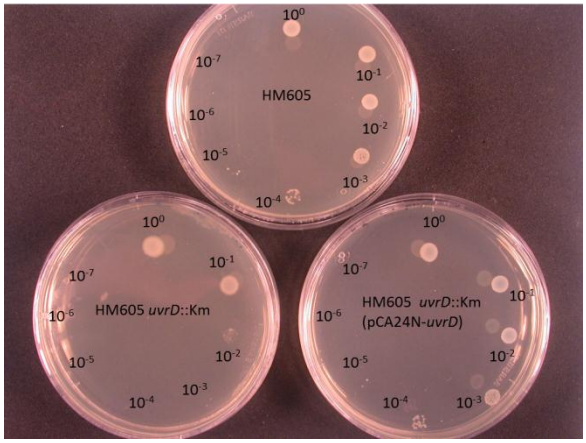


Figure 5.9: Decreased nitrosative stress tolerance of the *uvrD*::Km mutant. Stress tolerance of HM605 was compared to that of *uvrD* mutant and the complemented strain (*uvrD* (pCA24N-*uvrD*) on plates containing no stress (LB), low pH (100 mM MES pH 5.0), and reactive nitrogen species (100 mM MES pH 5.0 + 1 mM NaNO2). Shown is the result of a typical experiment. Experiments were carried out in triplicate.

These results indicate that UvrD is important for the response of HM605 to nitrosative and oxidative stresses and the likely explanation for the reduction in the intracellular numbers of the *uvrD* mutant in J774.A1 macrophage is that this mutant has become more sensitive to the ROS and NOS produced by the macrophage. As UvrD is involved in 2 DNA repair pathways, it also suggests a possible role for NER and MMR in the intramacrophage survival of HM605.

5.3.5 Nucleotide excision repair (NER) is required for intracellular replication of HM605

NER is a highly conserved DNA repair mechanism, responsible for the repair of bulky, helix distorting DNA damage induced by oxidative stress, UV radiation and cis-platinum (Kamenisch *et al.*, 2010). Two major roles for NER have been reported, 1) as a backup for when base-excision repair (BER) of small lesions becomes saturated, and 2) as a primary repair pathway for DNA damage produced by lipid peroxidation products (Kamenisch *et al.*, 2010). As previously stated ROS and NOS can directly attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation which alters membrane properties and disrupts membrane-bound proteins as well as attacking DNA resulting in damage (Cabiscol *et al.*, 2000). Inactivation of the *uvrD* gene has been found to reduce *M. tuberculosis* persistence in a mouse model of tuberculosis infection suggesting an important fitness role for the *uvrD* gene product (Curti *et al.*, 2007). In addition it has been established that the *uvrB* gene in *M. tuberculosis* also has a role during pathogenesis in mice (Darwin & Nathan, 2005).

Interestingly, the BER pathway is reported to be the most important cellular protection mechanism responding to oxidative DNA damage (Lu *et al.*, 2001). Reports suggest that *S. enterica* serovar Typhimurium initially prevents death by oxidative stress by preserving its genomic integrity through the function of BER, allowing the up-regulation of genes on SPI2 to further limit its exposure to macrophage oxidants (Suvarnapunya *et al.*, 2003; Suvarnapunya & Stein, 2005). Nitric oxide deaminates cytosine to uracil, adenine to hypoxanthine, and guanine to xanthine and oxidative stress results in formation of abasic sites, single- and double-stranded breaks in DNA and damage to nitrogenous bases such as the conversion of

guanine to 7,8-dihydro-8-oxoguanine (8-oxoG) (Kurthkoti *et al.*, 2008). Therefore DNA lesions are often subject to overlapping repair processes (Kurthkoti *et al.*, 2008). The next stage was to examine if mutations in other DNA repair pathways (specifically BER, NER and MMR) could affect the intramacrophagic numbers of HM605. The DNA repair genes *nfo* (BER), *uvrB* (NER) and *mutS* (MMR) were deleted in HM605 using P1 transduction and gentamicin protection assays were carried out. BER works to repair damaged DNA by multiple steps: removal of the damaged base by a cleavage of the glycosidic bond by a glycosylase enzyme generating an apyrimidinic or apurinic (AP) site; removal of the AP site by an AP endonuclease. Nfo is one of two (the other being XthA) 5' AP endonucleases in *E. coli* (Levin *et al.*, 1988; Motta *et al.*, 2010). As stated, the *uvrB* gene product takes part in the prokaryotic NER pathway, which removes bulky adducts on DNA and *mutS* is the master regulator of MMR (Lamers *et al.*, 2000). After 9h infection in a macrophage (corresponds to 6h post-gentamicin) no significant difference was observed in the intracellular levels of Δ*nfo*::Km and Δ*mutS*::Km compared to HM605. This would suggest that BER and MMR are not required for the intracellular survival of HM605. In contrast, deletion of *uvrB* significantly reduced the intracellular bacterial numbers at 1h and 6h post-gentamicin compared to HM605 to the same level as seen for mutations in *uvrD*. As both *uvrB* and *uvrD* are involved in NER, these findings indicate that the disruption of NER in HM605 significantly reduces the number of intracellular bacteria inside J774.A1 macrophage. As we have previously shown that the *uvrD* mutant is sensitive to commonly encountered DNA damaging agents such as ROS and acidified nitrite, these findings suggest a role for NER in the ability of HM605 to survive prolonged exposure to DNA-damaging conditions encountered in the phagosome of macrophage.

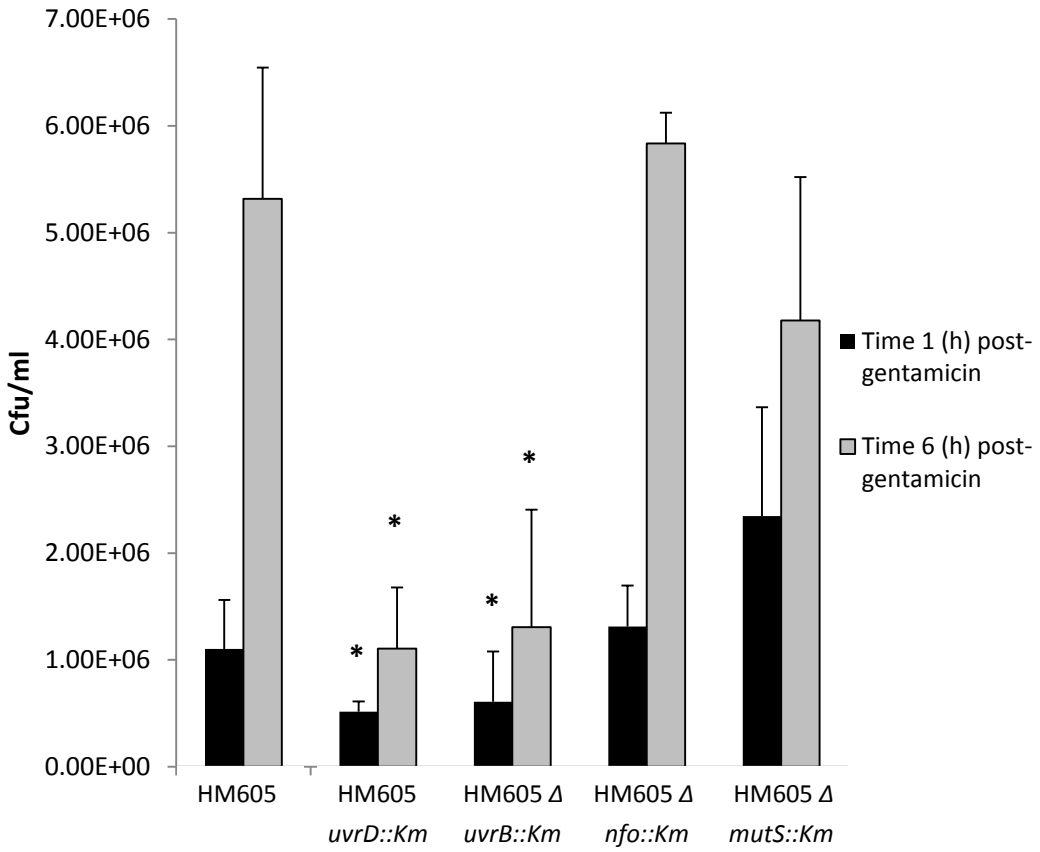


Figure 5.10: NER is required for replication in the macrophage. No significant difference in the numbers of intracellular bacteria was observed at 1h and 6hrs post-gentamicin between the wildtype strain and the *nfo* and *mutS* mutants. However, deletion of *uvrD* and *uvrB* significantly reduces the intracellular bacterial numbers at 1h and 6hrs post-gentamicin compared to wildtype HM605. Results are from triplicate determinations. For analysis of the significance of differences in uptake and survival within J774.A1 macrophage, Student's t-test was used for comparison of two groups of data ($*$ P-value ≤ 0.05).

5.4 Conclusion

Although studies have identified genes required for the intramacrophage replication of AIEC LF82 (Bringer *et al.*, 2005; Bringer *et al.*, 2007; Bringer *et al.*, 2011), to date little is known how other AIEC survive and replicate inside macrophage. Reactive oxidants are a primary antibacterial utilised by macrophage (Suvarnapunya *et al.*, 2003). ROS and NOS damage a range of macromolecules within the cell, but it is damage to DNA that is most likely to be lethal (Houghton *et al.*, 2012; Nathan & Shiloh, 2000). Moreover, several genetic factors have been identified in *S. enterica* serovar Typhimurium that are required for macrophage survival including resistance to ROS and NOS in the *Salmonella*-containing vacuole (SCV) and DNA repair (Suvarnapunya *et al.*, 2003; Suvarnapunya & Stein, 2005). Therefore, the initial aim of this chapter was to determine the role of resistance to oxidative stress during intramacrophage survival of the AIEC strain HM605. It is important to note that since transposon insertions were used to generate these mutants, this screen may have missed the identification of genes essential for survival during H_2O_2-stressed conditions or during growth.

A total of 3 non-essential genes with increased sensitivity to H_2O_2 were identified and the mutated genes were shown to be *katG* which encodes the bifunctional HPI hydroperoxidase enzyme, KatG (catalase), *hemF* which encodes the aerobically produced enzyme known as corpophyrinogen III oxidase and *uvrD*, whose product is a DNA helicase II involved in nucleotide excision repair (NER) and also mismatch repair (MMR) of DNA. All of these genes have previously been reported to be regulated by OxyR in a H_2O_2 -dependent manner in *E. coli* and have been previously identified as H_2O_2 -sensitive mutants in *E. coli* MC4100 (Mukhopadhyay & Schellhorn, 1997). Therefore, the identification of theses mutants in HM605 served to validate the screen as an effective means of selecting H_2O_2 sensitive mutants.

In vitro models, where the direct effect of H_2O_2 on bacterial cells was analysed, identified two separate modes of killing for *E. coli* (Linn & Imlay, 1987). At low concentrations of H_2O_2 ($\leq$2.5 mM), the main cause of *E. coli* cell death was due to DNA damage (Imlay & Linn, 1987; Linn & Imlay, 1987). At H_2O_2 concentrations of 10–50 mM, cell death resulted from cytotoxic effects due to hydroxyl radicals formed from H_2O_2 (Brudzynski *et al.*, 2011; Imlay & Linn, 1987). Thus, the identification of a H_2O_2 -sensitive mutant involved in DNA repair provides further

evidence for the interplay between the ROS scavenging system and DNA repair pathways. Moreover, studies of bacterial responses to oxidative stress found that several genes involved in DNA repair are important for resistance to H_2O_2 (Buchmeier *et al.*, 1993; Darwin & Nathan, 2005; Kurthkoti & Varshney, 2011; Levin *et al.*, 1988; Mertens *et al.*, 2005; Stohl & Seifert, 2006). Increased sensitivity to H_2O_2 was observed in both *recA* and *recBCD* mutants in *E. coli*, and this is thought to be due to these mutants being deficient in the induction of the SOS response, which provides a global response to DNA damage (Linn & Imlay, 1987). Furthermore, in *Neisseria gonorrhoea, recA* and the recombination associated gene *recN*, are both important for survival after H_2O_2 exposure (Stohl & Seifert, 2006).

Initial findings showed that it was only the intracellular survival of the *uvrD* mutant that was significantly ($P \leq 0.05$) reduced in J774.A1 macrophage compared to wild type (see fig 5.7). Further experimentation showed that the deletion of *uvrD* clearly increased the sensitivity of HM605 to a variety of chemical stress conditions, primarily ROS and NOS (see fig 5.9). These results indicate that HM605 is subjected to oxidative and nitrosative stress during phagocytosis supporting the hypothesis that the phagosome is mature. The importance of phagosomal maturation for intramacrophagic survival and/or replication of AIEC has already been reported (Bringer, 2006). Therefore, HM605 UvrD could potentially play an important role in promoting bacterial adaptation to the multiple stress conditions encountered in the phagocytic vacuole. Recent studies in *M. tuberculosis* showed that deletion of *uvrD* showed increased susceptibility to ROS and NOS and also showed decreased intracellular survival following infection of macrophages (Houghton *et al.*, 2012; Kurthkoti & Varshney, 2011). Together these findings suggest that a critical mechanism underpinning AIEC persistence in macrophages is the repair of DNA bases damaged by macrophage oxidants. However, given that every *E. coli* possesses *uvrD* but not every *E. coli* can grow inside macrophage, it is likely that AIEC may have evolved additional adaptation mechanisms to allow growth in the harsh environmental conditions of the mature phagosome.

Chapter 6.0: General discussion

In this study the adhesion, invasion, and intramacrophage replication capabilities (AIEC phenotype) of 84 *E. coli* strains were determined in order to identify the prevalence of the AIEC phenotype within the *E. coli* genus. It was demonstrated that approximately 5% of the *E. coli* genus exhibits the AIEC phenotype (see Chapter 3.0 Figure 3.2 and Table 3.2). This indicates that a significant proportion of *E. coli* strains are capable of adhering to and invading epithelial cells and undergoing intramacrophage replication. The significance of this is that *E. coli* exhibiting the AIEC phenotype in the compromised gut of CD patients could behave as opportunistic pathogens. Moreover, although a number of genes have been implicated in the pathogenesis of the AIEC it is important to note that these genes are also found in non-pathogenic *E. coli* K-12 strains (Barnich *et al.*, 2003; Rolhion *et al.*, 2007; Simonsen *et al.*, 2011). Analysis of the genome sequence of several strains of AIEC has also failed to identify any specific virulence factors associated with this group of bacteria although there does appear to be some similarity, at least at the genomic level, with UPEC and APEC (Miquel *et al.*, 2010). Furthermore, whilst the relationship between AIEC and ExPEC strains has been examined, further work is required to examine the genetic relationship between AIEC and other commensal and pathogenic strains of *E. coli*. Indeed, from this and other studies it now seems likely that AIEC are pathobionts i.e. commensals in a healthy gut but opportunistic pathogens when present in the compromised gut of a patient with CD.

The observation that intramacrophage replication is widespread in the *E. coli* genus led to the speculation as to what could be the selection or the driving force for the evolution of this phenotype? Could *E. coli* have adapted mechanisms to survive killing in protozoa which allowed them to survive phagocytosis by mammalian macrophage? Furthermore, what could this mean for the etiology of CD? Recent studies on the interactions between bacteria and protozoan predators have revealed new insights into how bacterial adaptive traits, such as cell surface properties and intracellular replication increase the survival of bacteria under grazing pressure (Casadevall, 2008; Matz *et al.*, 2004; Matz & Kjelleberg, 2005; Wildschutte *et al.*, 2004). The long co-evolutionary history between bacteria and protozoan predators

suggests that a series of adaptations ensuring bacterial survival could have emerged (Adiba *et al.*, 2010; Matz & Kjelleberg, 2005). The results presented in this study indicate a correlation between survival in macrophage and resistance to grazing by amoeba (Chapter 3 Table 3.3). Therefore, it could be hypothesised that amoeba harbouring AIEC-containing vesicles may be agents of transmission and environmental reservoirs of AIEC strains. This opens up the possibility of acquiring AIEC from the environment via drinking water and as AIEC could behave as opportunistic pathogens in the compromised gut of CD patients this could potentially add more complexity to the etiology of CD. In support of this hypothesis, studies have shown that strains of *Acanthamoeba* spp. have been found to produce *Legionella*-containing vesicles, which may be agents of transmission of Legionnaires Disease (Evstigneeva *et al.*, 2009). Interestingly, the selective pressure exerted by protozoan predators, in particular amoebae, is thought to have given rise to several facultative and obligate intracellular pathogens such as *M. avium* and *L. pneumophila* (Adiba *et al.*, 2010; Bozue & Johnson, 1996; Miltner & Bermudez, 2000). The fact that these intracellular pathogens exhibit intracellular survival within both amoebae and human macrophages by using similar mechanisms supports the coincidental evolution hypothesis that resistance to amoebae is an important prerequisite and, indeed, a driving force in the evolution of some bacteria, such as AIEC, as pathogens (Casadevall, 2008; Matz & Kjelleberg, 2005).

This is the first study to examine in depth, the molecular mechanisms of motility, cell association/invasion and intramacrophage survival/replication of an AIEC strain other than LF82. This study supports the hypothesis that AIEC are likely a diverse group of *E. coli* and possess diverse molecular mechanisms and virulence factors that contribute to the AIEC phenotype. For example, flagella and type 1 fimbriae have been shown to be essential invasion factors in LF82 (Barnich *et al.*, 2003; Eaves-Pyles, 2008). However, this study has shown that neither motility nor type 1 fimbriae are important for the invasion of epithelial cells by HM605 (see Chapter 4.0 fig 4.10 and fig 4.11). Moreover, the CpxA/R TCS has been shown not to be involved in the adhesion and invasion abilities of LF82 (Rolhion *et al.*, 2007). However, this study has shown that the CpxA/R TCS is involved in the invasion abilities of HM605 (see Chapter 4.0 fig. 4.6). This study has also added to the diverse molecular mechanisms of AIEC by identifying a potentially new invasin,

SinH whose expression is regulated by the CpxA/R TCS (see Chapter 4.0 fig. 4.20). The presence of SinH in a number of pathogenic *E. coli* and the AIEC strain LF82, suggest it may have an important role in the virulence of other *E. coli*. Together these results provide evidence to suggest that no one gene or virulence factor is involved in the AIEC phenotype and it appears the AIEC strains may have gone through different evolutionary history's acquiring various molecular mechanisms but ultimately culminating in the adhesion, invasion, and intramacrophage replication capabilities of AIEC.

In conclusion, this study supports the model that AIEC are pathobionts. Therefore these strains can be carried as commensals in healthy guts however, when the intestinal homeostasis is disrupted, such as in the compromised gut of CD patients, AIEC can behave as opportunistic pathogens and cause and/or contribute to disease. Several recent studies have identified environmental conditions that can result in pathobionts becoming pathogenic. For example it has been shown that dietary fats, by promoting changes in host bile acid composition, can drastically alter conditions for gut microbial assemblage, resulting in a dysbiosis that can disrupt immune homeostasis (Carvalho *et al.*, 2012; Devkota *et al.*, 2012). Such alterations in intestinal homeostasis could allow AIEC to behave as opportunistic pathogens. Similarly, a recent study described a sepsis-like disease that occurs upon gut injury in antibiotic-treated mice (Ayres *et al.*, 2012). Sepsis was attributed to the specific out-growth of a multidrug-resistant *E. coli* O21: H+ pathobiont. After antibiotic treatment this pathobiont (a normal member of the mouse gut microbiota) was able to establish a systemic infection that resulted in the activation of the inflammasome and the death of the host (Ayres *et al.*, 2012). Importantly in the absence of antibiotic treatment or gut injury no disease was apparent in mice carrying these bacteria in their gut. Further evidence can be seen with *Helicobacter hepaticus*, a Gram-negative bacterium of the intestinal microbiota. *H. hepaticus* promotes inflammation in animal models of colon cancer and causes a chronic inflammatory response similar to human IBD (Chow & Mazmanian, 2010; Erdman *et al.*, 2009). However, *H. hepaticus* only causes disease in immunocompromised animals that lack immune regulation and mount inflammatory responses toward intestinal bacteria (Erdman *et al.*, 2009). In a healthy individual, *H. hepaticus* limits colonization and intestinal inflammation, promoting a balanced relationship with the host (Chow &

Mazmanian, 2010). However, disruption of such balances contributes to human disorders such as IBD and colon cancer (Chow & Mazmanian, 2010). Together these studies also provide evidence for the convergent contributions of host genetic factors and epigenetic variables (i.e. the microbiota) to the cause of complex immunologic diseases such as CD (Chow & Mazmanian, 2010).

It has been recently proposed that CD-associated bacteria such as LF82 may not merely colonize the gut as a consequence of disease and/or be an aggravating factor of established disease but rather these bacteria might play an early role in disease pathogenesis, particularly as instigators of inflammation (Carvalho *et al.*, 2012). An important observation is that AIEC are also detected in healthy patients and do not appear to be pathogenic (Barnich & Darfeuille-Michaud, 2007b). This suggests that AIEC strains present in the compromised gut of their hosts may, under certain conditions, cross the epithelial barrier and replicate in macrophages. In doing so these bacteria may cause an increase in pro-inflammatory cytokines and thus contribute to the inflammation associated with CD. Therefore, AIEC may drive intestinal inflammation if their 'balance' with the host is altered. However, the challenge still remains to characterize the various molecular mechanisms that underpin the AIEC phenotype with the aim of identifying potential targets that might be useful in the treatment of AIEC-associated CD. Therapeutics which selectively target pathobionts may prove invaluable as a treatment for intestinal diseases such as CD.

Chapter 7.0: Bibliography

Abdallah, K. S., Cao, Y. & Wei, D. J. (2011). Epidemiologic Investigation of Extra-intestinal pathogenic E. coli (ExPEC) based on PCR phylogenetic group and fimH single nucleotide polymorphisms (SNPs) in China. *Int J Mol Epidemiol Genet* **2**, 339-353.

Adiba, S., Nizak, C., van Baalen, M., Denamur, E. & Depaulis, F. (2010). From grazing resistance to pathogenesis: the coincidental evolution of virulence factors. *PLoS ONE* **5**, e11882.

Aguirre, A., Cabeza, M. L., Spinelli, S. V., McClelland, M., Garcia Vescovi, E. & Soncini, F. C. (2006). PhoP-induced genes within Salmonella pathogenicity island 1. *J Bacteriol* **188**, 6889-6898.

Alm, E., Huang, K. & Arkin, A. (2006). The evolution of two-component systems in bacteria reveals different strategies for niche adaptation. *PLoS Comput Biol* **2**, e143.

Alsam, S., Jeong, S. R., Sissons, J., Dudley, R., Kim, K. S. & Khan, N. A. (2006). Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. *J Med Microbiol* **55**, 689-694.

Ambrose, N. S., Johnson, M., Burdon, D. W. & Keighley, M. R. (1984). Incidence of pathogenic bacteria from mesenteric lymph nodes and ileal serosa during Crohn's disease surgery. *Br J Surg* **71**, 623-625.

Amre, D. K., Mack, D., Israel, D., Morgan, K., Lambrette, P., Law, L., Grimard, G., Deslandres, C., Krupoves, A., Bucionis, V., Costea, I., Bissonauth, V., Feguery, H., D'Souza, S., Levy, E. & Seidman, E. G. (2008). Association between genetic variants in the IL-23R gene and early-onset Crohn's disease: results from a case-control and family-based study among Canadian children. *Am J Gastroenterol* **103**, 615-620.

Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J. & Hultgren, S. J. (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* **301**, 105-107.

Andrade, J. R., Da Veiga, V. F., De Santa Rosa, M. R. & Suassuna, I. (1989). An endocytic process in HEp-2 cells induced by enteropathogenic Escherichia coli. *J Med Microbiol* **28**, 49-57.

Andreoli, S. P., Trachtman, H., Acheson, D. W., Siegler, R. L. & Obrig, T. G. (2002). Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. *Pediatr Nephrol* **17**, 293-298.

Ansong, C., Yoon, H., Porwollik, S., Mottaz-Brewer, H., Petritis, B. O., Jaitly, N., Adkins, J. N., McClelland, M., Heffron, F. & Smith, R. D. (2009). Global systems-level analysis of Hfq and SmpB deletion mutants in Salmonella: implications for virulence and global protein translation. *PLoS ONE* **4**, e4809.

Aprikian, P., Tchesnokova, V., Kidd, B., Yakovenko, O., Yarov-Yarovoy, V., Trinchina, E., Vogel, V., Thomas, W. & Sokurenko, E. (2007). Interdomain interaction in the FimH

adhesin of Escherichia coli regulates the affinity to mannose. *J Biol Chem* **282**, 23437-23446.

Arifuzzaman, M., Maeda, M., Itoh, A. & other authors (2006). Large-scale identification of protein-protein interaction of Escherichia coli K-12. *Genome Res* **16**, 686-691.

Arnold, G. L., Beaves, M. R., Pryjdun, V. O. & Mook, W. J. (2002). Preliminary study of ciprofloxacin in active Crohn's disease. *Inflamm Bowel Dis* **8**, 10-15.

Asakura, H., Suzuki, K., Kitahora, T. & Morizane, T. (2008). Is there a link between food and intestinal microbes and the occurrence of Crohn's disease and ulcerative colitis? *J Gastroenterol Hepatol* **23**, 1794-1801.

Atlan, D., Coupat-Goutaland, B., Risler, A., Reyrolle, M., Souchon, M., Briolay, J., Jarraud, S., Doublet, P. & Pelandakis, M. (2012). Micriamoeba tesseris nov. gen. nov. sp.: A New Taxon of Free-living Small-sized Amoebae Non-permissive to Virulent Legionellae. *Protist*.

Autschbach, F., Eisold, S., Hinz, U., Zinser, S., Linnebacher, M., Giese, T., Loffler, T., Buchler, M. W. & Schmidt, J. (2005). High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* **54**, 944-949.

Ayres, J. S., Trinidad, N. J. & Vance, R. E. (2012). Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota. *Nat Med* **18**, 799-806.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. & Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006 0008.

Balfour Sartor, R. (2007). Bacteria in Crohn's disease: mechanisms of inflammation and therapeutic implications. *J Clin Gastroenterol* **41 Suppl 1**, S37-43.

Barker, J., Humphrey, T. J. & Brown, M. W. (1999). Survival of Escherichia coli O157 in a soil protozoan: implications for disease. *FEMS Microbiol Lett* **173**, 291-295.

Barnich, N., Boudeau, J., Claret, L. & Darfeuille-Michaud, A. (2003). Regulatory and functional co-operation of flagella and type 1 pili in adhesive and invasive abilities of AIEC strain LF82 isolated from a patient with Crohn's disease. *Mol Microbiol* **48**, 781-794.

Barnich, N., Bringer, M. A., Claret, L. & Darfeuille-Michaud, A. (2004). Involvement of lipoprotein NlpI in the virulence of adherent invasive Escherichia coli strain LF82 isolated from a patient with Crohn's disease. *Infect Immun* **72**, 2484-2493.

Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R. & Podolsky, D. K. (2005). Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition. *J Cell Biol* **170**, 21-26.

Barnich, N., Carvalho, F. A., Glasser, A. L., Darcha, C., Jantscheff, P., Allez, M., Peeters, H., Bommelaer, G., Desreumaux, P., Colombel, J. F. & Darfeuille-Michaud, A. (2007).

CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest* **117**, 1566-1574.

Barnich, N. & Darfeuille-Michaud, A. (2007a). Adherent-invasive Escherichia coli and Crohn's disease. *Curr Opin Gastroenterol* **23**, 16-20.

Barnich, N. & Darfeuille-Michaud, A. (2007b). Role of bacteria in the etiopathogenesis of inflammatory bowel disease. *World J Gastroenterol* **13**, 5571-5576.

Barnich, N. & Darfeuille-Michaud, A. (2010). Abnormal CEACAM6 expression in Crohn disease patients favors gut colonization and inflammation by adherent-invasive E. coli. *Virulence* **1**, 281-282.

Barnich, N., Boudeau, J., Claret, L., and Darfeuille-Michaud, A. (2003). Regulatory and functional co-operation of flagella and type 1 pili in adhesive and invasive abilities of AIEC strain LF82 isolated from a patient with Crohn's disease. *Mol Microbiol* **48**, 781-794.

Baumler, A. J., Kusters, J. G., Stojiljkovic, I. & Heffron, F. (1994). *Salmonella typhimurium* loci involved in survival within macrophages. *Infect Immun* **62**, 1623-1630.

Baumler, A. J., Tsolis, R. M. & Heffron, F. (1996). The lpf fimbrial operon mediates adhesion of Salmonella typhimurium to murine Peyer's patches. *Proc Natl Acad Sci U S A* **93**, 279-283.

Baysoy, G., Daar, G., Demir, H., Elmas, S. A., Haliloglu, M. & Besbas, N. (2011). Internal iliac vein thrombosis in pediatric Crohn's disease. *J Crohns Colitis* **5**, 57-59.

Beltran, B., Nos, P., Dasi, F. & other authors (2009). Mitochondrial dysfunction, persistent oxidative damage, and catalase inhibition in immune cells of naive and treated Crohn's disease. *Inflamm Bowel Dis* **16**, 76-86.

Berdal, B. P., Mehl, R., Meidell, N. K., Lorentzen-Styr, A. M. & Scheel, O. (1996). Field investigations of tularemia in Norway. *FEMS Immunol Med Microbiol* **13**, 191-195.

Bergstrand, O. & Hellers, G. (1983). Breast-feeding during infancy in patients who later develop Crohn's disease. *Scand J Gastroenterol* **18**, 903-906.

Bernardini, M. L., Fontaine, A. & Sansonetti, P. J. (1990). The two-component regulatory system ompR-envZ controls the virulence of Shigella flexneri. *J Bacteriol* **172**, 6274-6281.

Bernardini, M. L., Sanna, M. G., Fontaine, A. & Sansonetti, P. J. (1993). OmpC is involved in invasion of epithelial cells by Shigella flexneri. *Infect Immun* **61**, 3625-3635.

Bertrand, N., Houle, S., LeBihan, G., Poirier, E., Dozois, C. M. & Harel, J. (2010). Increased Pho regulon activation correlates with decreased virulence of an avian pathogenic Escherichia coli O78 strain. *Infect Immun* **78**, 5324-5331.

Beutin, L. & Martin, A. (2012). Outbreak of Shiga toxin-producing Escherichia coli (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *J Food Prot* **75**, 408-418.

Bijlsma, J. J. & Groisman, E. A. (2005). The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. *Mol Microbiol* **57**, 85-96.

Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T. & Brumell, J. H. (2006). Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem* **281**, 11374-11383.

Birrenbach, T. & Bocker, U. (2004). Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. *Inflamm Bowel Dis* **10**, 848-859.

Boirivant, M., Marini, M., Di Felice, G., Pronio, A. M., Montesani, C., Tersigni, R. & Strober, W. (1999). Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology* **116**, 557-565.

Bonen, D. K., Ogura, Y., Nicolae, D. L., Inohara, N., Saab, L., Tanabe, T., Chen, F. F., Foster, S.J., Duerr, R. H., Brant, S.R., Cho, J. H. & Nunez, G. (2003). Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology* **124**, 140-146.

Boudeau, J., Glasser, A. L., Masseret, E., Joly, B. & Darfeuille-Michaud, A. (1999). Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease. *Infect Immun*
67, 4499-4509.

Boudeau, J., Barnich, N. & Darfeuille-Michaud, A. (2001). Type 1 pili-mediated adherence of Escherichia coli strain LF82 isolated from Crohn's disease is involved in bacterial invasion of intestinal epithelial cells. *Mol Microbiol* **39**, 1272-1284.

Bower, J. M., Eto, D. S. & Mulvey, M. A. (2005). Covert operations of uropathogenic Escherichia coli within the urinary tract. *Traffic* **6**, 18-31.

Bozue, J. A. & Johnson, W. (1996). Interaction of Legionella pneumophila with Acanthamoeba castellanii: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infect Immun* **64**, 668-673.

Brand, S., Staudinger, T., Schnitzler, F., Pfennig, S., Hofbauer, K., Dambacher, J., Seiderer, J., Tillack, C., Konrad, A.,Crispin, A.,Goke, B.,Lohse, P. & Ochsenkuhn, T. (2005). The role of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and CARD15/NOD2 mutations in the susceptibility and phenotype of Crohn's disease. *Inflamm Bowel Dis* **11**, 645-652.

Brest, P., Lapaquette, P., Souidi, M., Lebrigand, K., Cesaro, A., Vouret-Craviari, V., Mari, B. Barbry, P., Mosnier, J. F., Hebuterne, X., Harel-Bellan, A., Mograbi, B., Darfeuille-Michaud, A. & Hofman, P. (2011). A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. *Nat Genet* **43**, 242-245.

Bridger, S., Lee, J. C., Bjarnason, I., Jones, J. E. & Macpherson, A. J. (2002). In siblings with similar genetic susceptibility for inflammatory bowel disease, smokers tend to develop Crohn's disease and non-smokers develop ulcerative colitis. *Gut* **51**, 21-25.

Bringer, M. A., Barnich, N., Glasser, A. L., Bardot, O. & Darfeuille-Michaud, A. (2005). HtrA stress protein is involved in intramacrophagic replication of adherent and invasive Escherichia coli strain LF82 isolated from a patient with Crohn's disease. *Infect Immun* **73**, 712-721.

Bringer, M. A., Glasser, A. L., Tung, C. H., Meresse, S. & Darfeuille-Michaud, A. (2006). The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages. *Cell Microbiol* **8**, 471-484.

Bringer, M. A., Rolhion, N., Glasser, A. L. & Darfeuille-Michaud, A. (2007). The oxidoreductase DsbA plays a key role in the ability of the Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 to resist macrophage killing. *J Bacteriol* **189**, 4860-4871.

Bringer, M. A., Billard, E., Glasser, A. L., Colombel, J. F. & Darfeuille-Michaud, A. (2011). Replication of Crohn's disease-associated AIEC within macrophages is dependent on TNF-alpha secretion. *Lab Invest* **92**, 411-419.

Bringer, M. A., Glasser, A.L., Tung, C.H., Meresse, S. and Darfeuille-Michaud, A. (2006). The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages. *Cell Microbiol* **8**, 471-484.

Bringer, M. A., Rolhion, N., Glasser, A.L., Darfeuille-Michaud, A. (2007). The Oxidoreductase DsbA plays a key role in the ability of the Crohns's disease-asscociated Adherent-Invasive Escherichia coli Strain LF82 to resist Macrophage Killing. *J Bacteriol* **189**, 4860-4871.

Brown, K., DeCoffe, D., Molcan, E. & Gibson, D. L. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. *Nutrients* **4**, 1095-1119.

Brudzynski, K., Abubaker, K., St Laurent, M. & Castle, A. (2011). Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Front Microbiol* **2**, 213.

Bruggemann, H., Cazalet, C. & Buchrieser, C. (2006). Adaptation of Legionella pneumophila to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. *Curr Opin Microbiol* **9**, 86-94.

Buchmeier, N. A., Lipps, C. J., So, M. Y. & Heffron, F. (1993). Recombination-deficient mutants of Salmonella typhimurium are avirulent and sensitive to the oxidative burst of macrophages. *Mol Microbiol* **7**, 933-936.

Burnens, A. P., Frey, A., Lior, H. & Nicolet, J. (1995). Prevalence and clinical significance of vero-cytotoxin-producing Escherichia coli (VTEC) isolated from cattle in herds with and without calf diarrhoea. *Zentralbl Veterinarmed B* **42**, 311-318.

Bush, T. G., Savidge, T. C., Freeman, T. C., Cox, H. J., Campbell, E. A., Mucke, L., Johnson, M. H. & Sofroniew, M. V. (1998). Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* **93**, 189-201.

Cabanes, D., Sousa, S., Cebria, A., Lecuit, M., Garcia-del Portillo, F. & Cossart, P. (2005). Gp96 is a receptor for a novel Listeria monocytogenes virulence factor, Vip, a surface protein. *EMBO J* **24**, 2827-2838.

Cabiscol, E., Tamarit, J. & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* **3**, 3-8.

Cadwell, K., Liu, J. Y., Brown, S. L., Miyoshi, H., Loh, J., Lennerz, J. K., Kishi, C., Kc, W., Carrero, J. A., Hunt, S., Stone, C. D., Brunt, E. M., Xavier, R. J., Sleckman, B. P., Li, E., Mizushima, N., Stappenbeck, T. S. & Virgin, H. W. th (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* **456**, 259-263.

Calkins, B. M. (1989). A meta-analysis of the role of smoking in inflammatory bowel disease. *Dig Dis Sci* **34**, 1841-1854.

Capra, E. J. & Laub, M. T. (2012). Evolution of Two-Component Signal Transduction Systems. *Annu Rev Microbiol* **66**, 325-347.

Caprilli, R. (2008). Why does Crohn's disease usually occur in terminal ileum? *J Crohns Colitis* **2**, 352-356.

Caprilli, R., Lapaquette, P. & Darfeuille-Michaud, A. (2010). Eating the enemy in Crohn's disease: an old theory revisited. *J Crohns Colitis* **4**, 377-383.

Cartun, R. W., Van Kruiningen, H. J., Pedersen, C. A. & Berman, M. M. (1993). An immunocytochemical search for infectious agents in Crohn's disease. *Mod Pathol* **6**, 212-219.

Carvalho, F. A., Barnich, N., Sivignon, A., Darcha, C., Chan, C. H., Stanners, C. P. & Darfeuille-Michaud, A. (2009). Crohn's disease adherent-invasive Escherichia coli colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. *J Exp Med* **206**, 2179-2189.

Carvalho, F. A., Koren, O., Goodrich, J. K., Johansson, M. E. V., Nalbantoglu, I., Aitken, J. D., Su, Y., Chassaing, B., Walters, W. A., González, A., Clemente, J. C., Cullender, T. C., Barnich, N., Darfeuille-Michaud, A., Vijay-Kumar, M., Knight, R., Ley, R. E., & Gewirtz, A.T. (2012). Transient Inability to Manage Proteobacteria Promotes Chronic Gut Inflammation in TLR5-Deficient Mice. *Cell Host Microbe* **12**, 139-152.

Casadevall, A. (2008). Evolution of intracellular pathogens. *Annu Rev Microbiol* **62**, 19-33.

Casalino, M., Prosseda, G., Barbagallo, M., Iacobino, A., Ceccarini, P., Latella, M. C., Nicoletti, M. & Colonna, B. (2009). Interference of the CadC regulator in the arginine-dependent acid resistance system of Shigella and enteroinvasive E. coli. *Int J Med Microbiol* **300**, 289-295.

Celli, J. (2006). Surviving inside a macrophage: the many ways of Brucella. *Res Microbiol* **157**, 93-98.

Ceresara, G., Fogagnolo, P., De Cilla, S., Panizzo, V., Danelli, P. G., Orzalesi, N. & Rossetti, L. (2010). Corneal involvement in Crohn's disease: an in vivo confocal microscopy study. *Cornea* **30**, 136-142.

Chassaing, B., Rolhion, N., de Vallee, A., Salim, S. Y.,Prorok-Hamon, M., Neut, C., Campbell, B. J., Soderholm, J. D., Hugot, J. P., Colombel, J. F. & Darfeuille-Michaud, A. (2011). Crohn disease--associated adherent-invasive E. coli bacteria target mouse and human Peyer's patches via long polar fimbriae. *J Clin Invest* **121**, 966-975.

Chassaing, B., Etienne-Mesmin, L., Bonnet, R. & Darfeuille-Michaud, A. (2012). Bile salts induce long polar fimbriae expression favouring Crohn's disease-associated adherent-invasive Escherichia coli interaction with Peyer's patches. *Environ Microbiol*.

Chaudhuri, R. R. & Henderson, I. R. (2012). The evolution of the Escherichia coli phylogeny. *Infect Genet Evol* **12**, 214-226.

Chiang, S. M. & Schellhorn, H. E. (2012). Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. *Arch Biochem Biophys* **525**, 161-169.

Chiodini, R. J., Van Kruiningen, H. J., Thayer, W. R., Merkal, R. S. & Coutu, J. A. (1984). Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified Mycobacterium species isolated from patients with Crohn's disease. *Dig Dis Sci* **29**, 1073-1079.

Cho, J. H. (2008). Inflammatory bowel disease: genetic and epidemiologic considerations. *World J Gastroenterol* **14**, 338-347.

Chow, J. & Mazmanian, S. K. (2010). A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell Host Microbe* **7**, 265-276.

Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell* **41**, 753-762.

Christman, M. F., Storz, G. & Ames, B. N. (1989). OxyR, a positive regulator of hydrogen peroxide-inducible genes in Escherichia coli and Salmonella typhimurium, is homologous to a family of bacterial regulatory proteins. *Proc Natl Acad Sci U S A* **86**, 3484-3488.

Cirillo, J. D., Falkow, S., Tompkins, L. S. & Bermudez, L. E. (1997). Interaction of Mycobacterium avium with environmental amoebae enhances virulence. *Infect Immun* **65**, 3759-3767.

Cirillo, S. L., Yan, L., Littman, M., Samrakandi, M. M. & Cirillo, J. D. (2002). Role of the Legionella pneumophila rtxA gene in amoebae. *Microbiology* **148**, 1667-1677.

Claret, L., Miquel, S., Vieille, N., Ryjenkov, D.A., Gomelsky, M., and Darfeuille Michaud, A. (2007). The flagellar sigma factor Flia regulates adhesion and invasion of crohn's disease-

associated escherichia coli via a C-di-GMP-dependent pathway. *J Biol Chem* **46**, 33275-33283.

Clarke, D. J., Chaudhuri, R. R., Martin, H. M., Campbell, B. J., Rhodes, J. M., Constantinidou, C., Pallen, M. J., Loman, N. J.,Cunningham, A. F., Browning, D. F. & Henderson, I. R. (2011). Complete genome sequence of the Crohn's disease-associated adherent-invasive Escherichia coli strain HM605. *J Bacteriol* **193**, 4540.

Clemens, D. L. & Horwitz, M. A. (1995). Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181**, 257-270.

Compan, I. & Touati, D. (1993). Interaction of six global transcription regulators in expression of manganese superoxide dismutase in Escherichia coli K-12. *J Bacteriol* **175**, 1687-1696.

Cookson, S. T. & Nataro, J. P. (1996). Characterization of HEp-2 cell projection formation induced by diffusely adherent Escherichia coli. *Microb Pathog* **21**, 421-434.

Cooney, R., Baker, J., Brain, O. & other authors (2009). NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* **16**, 90-97.

Cooper, T. F. (2007). Recombination speeds adaptation by reducing competition between beneficial mutations in populations of Escherichia coli. *PLoS Biol* **5**, e225.

Corrao, G., Tragnone, A., Caprilli, R. & other authors (1998). Risk of inflammatory bowel disease attributable to smoking, oral contraception and breastfeeding in Italy: a nationwide case-control study. Cooperative Investigators of the Italian Group for the Study of the Colon and the Rectum (GISC). *Int J Epidemiol* **27**, 397-404.

Crepin, S., Chekabab, S. M., Le Bihan, G., Bertrand, N., Dozois, C. M. & Harel, J. (2011). The Pho regulon and the pathogenesis of Escherichia coli. *Vet Microbiol* **153**, 82-88.

Crohn, B. B., Ginzburg, L. & Oppenheimer, G. D. (1984). Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. By Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer. *JAMA* **251**, 73-79.

Crohn. B.B, Ginzburg, L. & Oppenheimer, G. D. (1932). Regional ileitis; a pathologic and clinical entity. *JAMA* **99**, 1323-1329.

Csongei, V., Jaromi, L., Safrany, E. & other authors (2010). Interaction of the major inflammatory bowel disease susceptibility alleles in Crohn's disease patients. *World J Gastroenterol* **16**, 176-183.

Curti, E., Smerdon, S. J. & Davis, E. O. (2007). Characterization of the helicase activity and substrate specificity of Mycobacterium tuberculosis UvrD. *J Bacteriol* **189**, 1542-1555.

Cuthbert, A. P., Fisher, S. A., Mirza, M. M., King, K., Hampe, J., Croucher, P. J., Mascheretti, S., Sanderson, J., Forbes, A., Mansfield, J., Schreiber, S., Lewis, C. M. &

Mathew, C. G. (2002). The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology* **122**, 867-874.

Czeczulin, J. R., Balepur, S., Hicks, S., Phillips, A., Hall, R., Kothary, M. H., Navarro-Garcia, F. & Nataro, J. P. (1997). Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative Escherichia coli. *Infect Immun* **65**, 4135-4145.

D'Inca, R., Sturniolo, G., Cassaro, M., di Pace, C., Longo, G., Callegari, I. & Rugge, M. (1998). Prevalence of upper gastrointestinal lesions and Helicobacter pylori infection in Crohn's disease. *Dig Dis Sci* **43**, 988-992.

Dailey, F. E. & Berg, H. C. (1993). Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. *Proc Natl Acad Sci USA*
90, 1043-1047.

Daly, M. J., Rioux, J. D., Schaffner, S. F., Hudson, T. J. & Lander, E. S. (2001). High-resolution haplotype structure in the human genome. *Nat Genet* **29**, 229-232.

Dalziel, T. (1913). Chronic intestinal enteritis. *BMJ* **2**, 1068-1070.

Danese, P. N. & Silhavy, T. J. (1998). CpxP, a stress-combative member of the Cpx regulon. *J Bacteriol* **180**, 831-839.

Darfeuille-Michaud, A. (2002). Adherent-invasive Escherichia coli: a putative new E. coli pathotype associated with Crohn's disease. *Int J Med Microbiol* **292**, 185-193.

Darfeuille-Michaud, A., Boudeau, J., Bulois, P., Neut, C., Glasser, A.L., Barnich, N., Bringer, M.A., Swidsinski, A., Beaugerie, L., and Colombel, J.F. (2004). High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology*
127, 412-421.

Darfeuille-Michaud, A., Neut, C., Barnich, N., Lederman, E., Di Martino, P., Desreumaux, P., Gambiez, L., Joly, B., Cortot, A., and Colombel, J.F. (1998). Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* **115**, 1405-1413.

Darwin, K. H. & Nathan, C. F. (2005). Role for nucleotide excision repair in virulence of Mycobacterium tuberculosis. *Infect Immun* **73**, 4581-4587.

Dawson, K. G., Emerson, J. C. & Burns, J. L. (1999). Fifteen years of experience with bacterial meningitis. *Pediatr Infect Dis J* **18**, 816-822.

De Wulf, P., Kwon, O. & Lin, E. C. (1999). The CpxRA signal transduction system of Escherichia coli: growth-related autoactivation and control of unanticipated target operons. *J Bacteriol* **181**, 6772-6778.

Denizot, J., Sivignon, A., Barreau, F., Darcha, C., Chan, H. F., Stanners, C. P., Hofman, P., Darfeuille-Michaud, A. & Barnich, N. (2011). Adherent-invasive Escherichia coli induce

claudin-2 expression and barrier defect in CEABAC10 mice and Crohn's disease patients. *Inflamm Bowel Dis* **18**, 294-304.

Dersch, P. & Isberg, R. R. (1999). A region of the Yersinia pseudotuberculosis invasin protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. *EMBO J* **18**, 1199-1213.

Desjardins, M. (1995). Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. *Trends Cell Biol* **5**, 183-186.

Desjardins, P., Picard, B., Kaltenbock, B., Elion, J. & Denamur, E. (1995). Sex in Escherichia coli does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. *J Mol Evol* **41**, 440-448.

Detweiler, C. S., Monack, D.M., Brodsky, I.E., Mathew, H., and Falkow, S. (2003). virK, somA and rcsC are important for systemic Salmonella enterica serovar Typhimurium infection and cationic peptide resistance. *Mol Microbiol* **48**, 385-400.

Devkota, S., Wang, Y., Musch, M. W., Leone, V., Fehlner-Peach, H., Nadimpalli, A., Antonopoulos, D. A., Jabri, B. & Chang, E. B. (2012). Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* **487**, 104-108.

Dhakal, B. K., Kulesus, R. R. & Mulvey, M. A. (2008). Mechanisms and consequences of bladder cell invasion by uropathogenic Escherichia coli. *Eur J Clin Invest* **38 Suppl 2**, 2-11.

Di Meglio, P., Di Cesare, A., Laggner, U., Chu, C. C., Napolitano, L., Villanova, F., Tosi, I. Capon, F., Trembath, R. C., Peris, K. & Nestle, F. O. (2011). The IL23R R381Q gene variant protects against immune-mediated diseases by impairing IL-23-induced Th17 effector response in humans. *PLoS ONE* **6**, e17160.

Diamant, E., Palti, Y., Gur-Arie, R., Cohen, H., Hallerman, E. M. & Kashi, Y. (2004). Phylogeny and strain typing of Escherichia coli, inferred from variation at mononucleotide repeat loci. *Appl Environ Microbiol* **70**, 2464-2473.

Didelot, X., Meric, G., Falush, D. & Darling, A. E. (2012). Impact of homologous and non-homologous recombination in the genomic evolution of Escherichia coli. *BMC Genomics* **13**, 256.

Dogan, B., Scherl, E., Bosworth, B., Yantiss, R., Altier, C., McDonough, P. L., Jiang, Z. D., Dupont, H. L., Garneau, P., Harel, J., Rishniw, M. & Simpson, K. W (2012). Multidrug resistance is common in Escherichia coli associated with ileal Crohn's disease. *Inflamm Bowel Dis*.

Donnenberg, M. S., Donohue-Rolfe, A. & Keusch, G. T. (1989). Epithelial cell invasion: an overlooked property of enteropathogenic Escherichia coli (EPEC) associated with the EPEC adherence factor. *J Infect Dis* **160**, 452-459.

Donnenberg, M. S., Donohue-Rolfe, A. & Keusch, G. T. (1990). A comparison of HEp-2 cell invasion by enteropathogenic and enteroinvasive Escherichia coli. *FEMS Microbiol Lett* **57**, 83-86.

Donnenberg, M. S., Zhang, H.-Z. & Stone, K. D. (1997). Biogenesis of the bundle-forming pilus of enteropathogenic *Escherichia coli*: reconstitution of fimbriae in recombinant *E. coli* and role of DsbA in pilin stability - a review. *Gene* **192**, 33-38.

Dorel, C., Lejeune, P. & Rodrigue, A. (2006). The Cpx system of Escherichia coli, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? *Res Microbiol* **157**, 306-314.

Dubbs, J. M. & Mongkolsuk, S. (2012). Peroxide Sensing Transcriptional Regulators in Bacteria. *J Bacteriol*.

Dubinsky, M. C., Wang, D., Picornell, Y., Wrobel, I., Katzir, L., Quiros, A., Dutridge, D. Wahbeh, G., Silber, G., Bahar, R., Mengesha, E., Targan, S. R., Taylor, K. D. & Rotter, J. I. (2007). IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflamm Bowel Dis* **13**, 511-515.

Duerr, R. H., Taylor, K. D., Brant, S. R., Rioux, J. D., Silverberg, M. S., Daly, M. J., Steinhart, A. H., Abraham, C., Regueiro, M., Griffiths, A., Dassopoulos, T., Bitton, A., Yang, H., Targan, S., Datta, L. W., Kistner, E. O., Schumm, L. P., Lee, A. T., Gregersen, P. K., Barmada, M. M., Rotter, J. I., Nicolae, D. L. & Cho, J. H. (2006). A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* **314**, 1461-1463.

Eaves-Pyles, T., Allen, C.A., Taormina, J., Swidsinki, A., Tutt, C.B., Jezek, G.E., Islas-Islas, M. and Torres, A.G. (2008). (2008). Escherichia coli isolated from a Crohn's Disease patient adheres, invades and induces inflammatory responses in polarized epithelial cells. *Intern Journal of Med Microbiol*
298, 397-409.

Eckburg, P. B. & Relman, D. A. (2007). The role of microbes in Crohn's disease. *Clin Infect Dis* **44**, 256-262.

Eder, C. (2009). Mechanisms of interleukin-1beta release. *Immunobiology* **214**, 543-553.

Eggert, U. S., Ruiz, N., Falcone, B. V., Branstrom, A. A., Goldman, R. C., Silhavy, T. J. & Kahne, D. (2001). Genetic basis for activity differences between vancomycin and glycolipid derivatives of vancomycin. *Science* **294**, 361-364.

Eguchi, Y., Itou, J., Yamane, M., Demizu, R., Yamato, F., Okada, A., Mori, H., Kato, A. & Utsumi, R. (2007). B1500, a small membrane protein, connects the two-component systems EvgS/EvgA and PhoQ/PhoP in Escherichia coli. *Proc Natl Acad Sci U S A* **104**, 18712-18717.

Ekbom, A., Wakefield, A. J., Zack, M. & Adami, H. O. (1994). Perinatal measles infection and subsequent Crohn's disease. *Lancet* **344**, 508-510.

Elzer, P. H., Phillips, R. W., Robertson, G. T. & Roop, R. M., 2nd (1996). The HtrA stress response protease contributes to resistance of Brucella abortus to killing by murine phagocytes. *Infect Immun* **64**, 4838-4841.

Erdman, S. E., Rao, V. P., Poutahidis, T. & other authors (2009). Nitric oxide and TNF-alpha trigger colonic inflammation and carcinogenesis in Helicobacter hepaticus-infected, Rag2-deficient mice. *Proc Natl Acad Sci U S A* **106**, 1027-1032.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. D. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. *Molecular Microbiology* **47**, 103-118.

Eto, D. S., Sundsbak, J. L. & Mulvey, M. A. (2006). Actin-gated intracellular growth and resurgence of uropathogenic Escherichia coli. *Cell Microbiol* **8**, 704-717.

Eto, D. S., Gordon, H.B., Dhakal, B.K., Jones, T.A., and Mulvey, M.A. (2008). Clathrin, AP-2 and the NPXYP-binding subset of alternate endocytic adaptors facilitate FimH-mediated bacterial invasion of host cells. *Cell Microbiol* **12**, 2553-2567

Evstigneeva, A., Raoult, D., Karpachevskiy, L. & La Scola, B. (2009). Amoeba co-culture of soil specimens recovered 33 different bacteria, including four new species and Streptococcus pneumoniae. *Microbiology* **155**, 657-664.

Farr, S. B. & Kogoma, T. (1991). Oxidative stress responses in Escherichia coli and Salmonella typhimurium. *Microbiol Rev* **55**, 561-585.

Fava, F. & Danese, S. (2011). Intestinal microbiota in inflammatory bowel disease: friend of foe? *World J Gastroenterol* **17**, 557-566.

Feuerstein, O., Moreinos, D. & Steinberg, D. (2006). Synergic antibacterial effect between visible light and hydrogen peroxide on Streptococcus mutans. *J Antimicrob Chemother* **57**, 872-876.

Finlay, B. B. & Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**, 718-725.

Fiocchi, C. (2005). Inflammatory bowel disease pathogenesis: therapeutic implications. *Chin J Dig Dis* **6**, 6-9.

Flannagan, R. S., Cosio, G. & Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* **7**, 355-366.

Foxman, B. & Brown, P. (2003). Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. *Infect Dis Clin North Am* **17**, 227-241.

Frank, D. N., St. Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N. and Pace, N.R. (2007). Molecular-Phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *PNAS* **104**, 13780-13785.

Friswell, M., Campbell, B. & Rhodes, J. (2010). The role of bacteria in the pathogenesis of inflammatory bowel disease. *Gut Liver* **4**, 295-306.

Fritz, T., Niederreiter, L., Adolph, T., Blumberg, R. S. & Kaser, A. (2011). Crohn's disease: NOD2, autophagy and ER stress converge. *Gut* **60**, 1580-1588.

Fujimura, Y., Kamoi, R. & Iida, M. (1996). Pathogenesis of aphthoid ulcers in Crohn's disease: correlative findings by magnifying colonoscopy, electron microscopy, and immunohistochemistry. *Gut* **38**, 724-732.

Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T. & Yoshimori, T. (2008). The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell* **19**, 2092-2100.

Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., Fiocchi, C. & Strober, W. (1996). Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* **157**, 1261-1270.

Fyderek, K., Strus, M., Kowalska-Duplaga, K., Gosiewski, T., Wedrychowicz, A., Jedynak, Wasowicz, U., Sladek, M., Pieczarkowski, S., Adamski, P., Kochan, P. & Heczko, P. B. (2009). Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease. *World J Gastroenterol* **15**, 5287-5294.

Galeazzi, F., Blennerhassett, P. A., Qiu, B., O'Byrne, P. M. & Collins, S. M. (1999). Cigarette smoke aggravates experimental colitis in rats. *Gastroenterology* **117**, 877-883.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3**, 710-720.

Gao, R., Tao, Y. & Stock, A. M. (2008). System-level mapping of Escherichia coli response regulator dimerization with FRET hybrids. *Mol Microbiol* **69**, 1358-1372.

Garcia-Lafuente, A., Antolin, M., Guarner, F., Crespo, E. & Malagelada, J. R. (2001). Modulation of colonic barrier function by the composition of the commensal flora in the rat. *Gut* **48**, 503-507.

Garcia de Tena, J., Manzano, L., Leal, J. C., San Antonio, E., Sualdea, V. & Alvarez-Mon, M. (2006). Distinctive pattern of cytokine production and adhesion molecule expression in peripheral blood memory CD4+ T cells from patients with active Crohn's disease. *J Clin Immunol* **26**, 233-242.

Gaulin, C., Levac, E., Ramsay, D., Dion, R., Ismail, J., Gingras, S. & Lacroix, A. (2012). Escherichia coli O157:H7 Outbreak Linked to Raw Milk Cheese in Quebec, Canada: Use of Exact Probability Calculation and CaseCase Study Approaches to Foodborne Outbreak Investigation. *J Food Prot* **75**, 812-818.

Gazouli, M., Pachoula, I., Panayotou, I., Mantzaris, G., Chrousos, G., Anagnou, N. P. & Roma-Giannikou, E. (2010). NOD2/CARD15, ATG16L1 and IL23R gene polymorphisms and childhood-onset of Crohn's disease. *World J Gastroenterol* **16**, 1753-1758.

Gellatly, S. L., Needham, B., Madera, L., Trent, M. S. & Hancock, R. E. (2012). Pseudomonas aeruginosa PhoP-PhoQ is induced upon interaction with epithelial cells and controls cytotoxicity and inflammation. *Infect Immun*.

Gerken, H., Charlson, E. S., Cicirelli, E. M., Kenney, L. J. & Misra, R. (2009). MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. *Mol Microbiol* **72**, 1408-1422.

Giaffer, M. H., Holdsworth, C. D. & Duerden, B. I. (1992). Virulence properties of Escherichia coli strains isolated from patients with inflammatory bowel disease. *Gut* **33**, 646-650.

Gionchetti, P., Rizzello, F., Morselli, C., Romagnoli, R. & Campieri, M. (2005). Management of inflammatory bowel disease: does rifaximin offer any promise? *Chemotherapy* **51 Suppl 1**, 96-102.

Giron, J. A., Ho, A. S. & Schoolnik, G. K. (1991). An inducible bundle-forming pilus of enteropathogenic Escherichia coli. *Science* **254**, 710-713.

Glasser, A. L., Boudeau, J., Barnich, N., Perruchot, M. H., Colombel, J. F. & Darfeuille-Michaud, A. (2001). Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infect Immun* **69**, 5529-5537.

Goldberg, M. B., Barzu, O., Parsot, C. & Sansonetti, P. J. (1993). Unipolar localization and ATPase activity of IcsA, a Shigella flexneri protein involved in intracellular movement. *J Bacteriol* **175**, 2189-2196.

Golubeva, Y. A. & Slauch, J. M. (2006). Salmonella enterica serovar Typhimurium periplasmic superoxide dismutase SodCI is a member of the PhoPQ regulon and is induced in macrophages. *J Bacteriol* **188**, 7853-7861.

Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A. & Utsumi, R. (2010). Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr Opin Microbiol* **13**, 232-239.

Grant, S. F., Baldassano, R. N. & Hakonarson, H. (2008). Classification of genetic profiles of Crohn's disease: a focus on the ATG16L1 gene. *Expert Rev Mol Diagn* **8**, 199-207.

Greub, G. & Raoult, D. (2004). Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* **17**, 413-433.

Griffin, P. M., Olmstead, L. C. & Petras, R. E. (1990). Escherichia coli O157:H7-associated colitis. A clinical and histological study of 11 cases. *Gastroenterology* **99**, 142-149.

Gunther, N. W. t., Lockatell, V., Johnson, D. E. & Mobley, H. L. (2001). In vivo dynamics of type 1 fimbria regulation in uropathogenic Escherichia coli during experimental urinary tract infection. *Infect Immun* **69**, 2838-2846.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I. & Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766.

Guttman, D. S. & Dykhuizen, D. E. (1994). Clonal divergence in Escherichia coli as a result of recombination, not mutation. *Science* **266**, 1380-1383.

Hacker, J. & Kaper, J. B. (2000). Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol* **54**, 641-679.

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F. M., Briggs, J., Gunther, S., Prescott, N. J., Onnie, C. M., Hasler, R., Sipos, B., Folsch, U. R., Lengauer, T., Platzer, M., Mathew, C. G., Krawczak, M. & Schreiber, S. (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* **39**, 207-211.

Harris, N. B. & Barletta, R. G. (2001). Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine. *Clin Microbiol Rev* **14**, 489-512.

Hase, K., Kawano, K., Nochi, T., Pontes, G. S., Fukuda, S., Ebisawa, M., Kadokura, K., Tobe, T., Fujimura, Y., Kawano, S., Yabashi, A., Waguri, S., Nakato, G., Kimura, S., Murakami, T., Iimura, M., Hamura, K., Fukuoka, S., Lowe, A. W., Itoh, K., Kiyono, H. & Ohno, H. (2009). Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature* **462**, 226-230.

Hassett, D. J. & Cohen, M. S. (1989). Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *FASEB J* **3**, 2574-2582.

Heimann, T. M., Greenstein, A. J., Lewis, B., Kaufman, D., Heimann, D. M. & Aufses, A. H., Jr. (1998). Comparison of primary and reoperative surgery in patients with Crohns disease. *Ann Surg* **227**, 492-495.

Heras, B., Shouldice, S. R., Totsika, M., Scanlon, M. J., Schembri, M. A. & Martin, J. L. (2009). DSB proteins and bacterial pathogenicity. *Nat Rev Microbiol* **7**, 215-225.

Herbert, E. E., Cowles, K. N. & Goodrich-Blair, H. (2007). CpxRA regulates mutualism and pathogenesis in Xenorhabdus nematophila. *Appl Environ Microbiol* **73**, 7826-7836.

Hermiston, M. L. & Gordon, J. I. (1995). Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* **270**, 1203-1207.

Herren, C. D., Mitra, A., Palaniyandi, S. K., Coleman, A., Elankumaran, S. & Mukhopadhyay, S. (2006). The BarA-UvrY two-component system regulates virulence in avian pathogenic Escherichia coli O78:K80:H9. *Infect Immun* **74**, 4900-4909.

Hicks, S., Candy, D. C. & Phillips, A. D. (1996). Adhesion of enteroaggregative Escherichia coli to pediatric intestinal mucosa in vitro. *Infect Immun* **64**, 4751-4760.

Hodge, S., Hodge, G., Ahern, J., Jersmann, H., Holmes, M. & Reynolds, P. N. (2007). Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **37**, 748-755.

Holdstock, G., Savage, D., Harman, M. & Wright, R. (1984). Should patients with inflammatory bowel disease smoke? *Br Med J (Clin Res Ed)* **288**, 362.

Holt, K. E., Thomson, N. R., Wain, J., Langridge, G. C., Hasan, R., Bhutta, Z. A., Quail, M. A., Norbertczak, H., Walker, D., Simmonds, M., White, B., Bason, N., Mungall, K., Dougan, G.

& Parkhill, J. (2009). Pseudogene accumulation in the evolutionary histories of Salmonella enterica serovars Paratyphi A and Typhi. *BMC Genomics* **10**, 36.

Hommais, F., Krin, E., Coppee, J. Y., Lacroix, C., Yeramian, E., Danchin, A. & Bertin, P. (2004). GadE (YhiE): a novel activator involved in the response to acid environment in Escherichia coli. *Microbiology* **150**, 61-72.

Horwitz, M. A. (1983). Formation of a novel phagosome by the Legionnaires' disease bacterium (Legionella pneumophila) in human monocytes. *J Exp Med* **158**, 1319-1331.

Houghton, J., Townsend, C., Williams, A. R., Rodgers, A., Rand, L., Walker, K. B., Bottger, E. C., Springer, B. & Davis, E. O. (2012). Important role for Mycobacterium tuberculosis UvrD1 in pathogenesis and persistence apart from its function in nucleotide excision repair. *J Bacteriol* **194**, 2916-2923.

Hruz, P. & Eckmann, L. (2011). Innate immune defence: NOD2 and autophagy in the pathogenesis of Crohn's disease. *Swiss Med Wkly* **140**, w13135.

Hugot, J. P., Chamaillard, M., Zouali, H. & other authors (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599-603.

Humphreys, S., Rowley, G., Stevenson, A., Anjum, M. F., Woodward, M. J., Gilbert, S., Kormanec, J. & Roberts, M. (2004). Role of the two-component regulator CpxAR in the virulence of Salmonella enterica serotype Typhimurium. *Infect Immun* **72**, 4654-4661.

Hung, D. L., Raivio, T. L., Jones, C. H., Silhavy, T. J. & Hultgren, S. J. (2001). Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili. *EMBO J* **20**, 1508-1518.

Huws, S. A., Morley, R. J., Jones, M. V., Brown, M. R. & Smith, A. W. (2008). Interactions of some common pathogenic bacteria with Acanthamoeba polyphaga. *FEMS Microbiol Lett* **282**, 258-265.

Imlay, J. A. & Linn, S. (1987). Mutagenesis and stress responses induced in Escherichia coli by hydrogen peroxide. *J Bacteriol* **169**, 2967-2976.

Ionescu, M. & Belkin, S. (2009). Overproduction of exopolysaccharides by an Escherichia coli K-12 rpoS mutant in response to osmotic stress. *Appl Environ Microbiol* **75**, 483-492.

Iwanaga, Y., Davey, M. P., Martin, T. M., Planck, S. R., DePriest, M. L., Baugh, M. M., Suing, C. M. & Rosenbaum, J. T. (2003). Cloning, sequencing and expression analysis of the mouse NOD2/CARD15 gene. *Inflamm Res* **52**, 272-276.

Jabado, N., Jankowski, A., Dougaparsad, S., Picard, V., Grinstein, S. & Gros, P. (2000). Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. *J Exp Med* **192**, 1237-1248.

Jackson, S. A., Mammel, M. K., Patel, I. R., Mays, T., Albert, T. J., LeClerc, J. E. & Cebula, T. A. (2007). Interrogating genomic diversity of E. coli O157:H7 using DNA tiling arrays. *Forensic Sci Int* **168**, 183-199.

Jackson, S. A., Patel, I. R., Barnaba, T., LeClerc, J. E. & Cebula, T. A. (2011). Investigating the global genomic diversity of Escherichia coli using a multi-genome DNA microarray platform with novel gene prediction strategies. *BMC Genomics* **12**, 349.

Jacob-Dubuisson, F., Pinkner, J., Xu, Z., Striker, R., Padmanhaban, A. & Hultgren, S. J. (1994). PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA. *Proc Natl Acad Sci USA*
91, 11552-11556.

Jerse, A. E., Yu, J., Tall, B. D. & Kaper, J. B. (1990). A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci U S A* **87**, 7839-7843.

Jones, C. H., Pinkner, J. S., Roth, R., Heuser, J., Nicholes, A. V., Abraham, S. N. & Hultgren, S. J. (1995). FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. *Proc Natl Acad Sci U S A* **92**, 2081-2085.

Jones, C. H., Danese, P. N., Pinkner, J. S., Silhavy, T. J. & Hultgren, S. J. (1997). The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. *EMBO J* **16**, 6394-6406.

Jones, C. J., Macnab, R. M., Okino, H. & Aizawa, S. (1990). Stoichiometric analysis of the flagellar hook-(basal-body) complex of Salmonella typhimurium. *J Mol Biol* **212**, 377-387.

Kadurugamuwa, J. L. & Beveridge, T. J. (1998). Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using Shigella flexneri membrane vesicles. *Antimicrob Agents Chemother* **42**, 1476-1483.

Kagnoff, M. F. & Eckmann, L. (1997). Epithelial cells as sensors for microbial infection. *J Clin Invest* **100**, 6-10.

Kamenisch, Y., Fousteri, M., Knoch, J. & other authors (2010). Proteins of nucleotide and base excision repair pathways interact in mitochondria to protect from loss of subcutaneous fat, a hallmark of aging. *J Exp Med* **207**, 379-390.

Kaper, J. B., Nataro, J.P., and Mobley, H.L.T. (2004). Pathogenic Escherichia coli. *Nat Rev Microbiol* **2:**, 123-140.

Kaser, A. & Blumberg, R. S. (2008). Adaptive immunity in inflammatory bowel disease: state of the art. *Curr Opin Gastroenterol* **24**, 455-461.

Kaserer, W. A., Jiang, X., Xiao, Q., Scott, D. C., Bauler, M., Copeland, D., Newton, S. M. & Klebba, P. E. (2008). Insight from TonB hybrid proteins into the mechanism of iron transport through the outer membrane. *J Bacteriol* **190**, 4001-4016.

Kavathas, P. B., Boeras, C. M., Mulla, M. J. & Abrahams, V. M. (2012). Nod1, but not the ASC inflammasome, contributes to induction of IL-1beta secretion in human trophoblasts after sensing of Chlamydia trachomatis. *Mucosal Immunol*.

Kawada-Matsuo, M., Yoshida, Y., Nakamura, N. & Komatsuzawa, H. (2011). Role of two-component systems in the resistance of Staphylococcus aureus to antibacterial agents. *Virulence* **2**, 427-430.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A. & Finlay, B. B. (1997). Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**, 511-520.

Khan, A., Datta, S., Das, S. C., Ramamurthy, T., Khanam, J., Takeda, Y., Bhattacharya, S. K. & Nair, G. B. (2003). Shiga toxin producing Escherichia coli infection: current progress & future challenges. *Indian J Med Res* **118**, 1-24.

Khan, M. T., Duncan, S. H., Stams, A. J., van Dijl, J. M., Flint, H. J. & Harmsen, H. J. (2012). The gut anaerobe Faecalibacterium prausnitzii uses an extracellular electron shuttle to grow at oxic-anoxic interphases. *ISME J*.

King, C. H., Shotts, E. B., Jr., Wooley, R. E. & Porter, K. G. (1988). Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Appl Environ Microbiol* **54**, 3023-3033.

Kingsley, R. A., Humphries, A. D., Weening, E. H., De Zoete, M. R., Winter, S., Papaconstantinopoulou, A., Dougan, G. & Baumler, A. J. (2003). Molecular and phenotypic analysis of the CS54 island of Salmonella enterica serotype typhimurium: identification of intestinal colonization and persistence determinants. *Infect Immun* **71**, 629-640.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H. & Mori, H. (2005). Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA Res* **12**, 291-299.

Klemm, P. (1986). Two regulatory fim genes, fimB and fimE, control the phase variation of type 1 fimbriae in Escherichia coli. *EMBO J* **5**, 1389-1393.

Klemm, P. & Christiansen, G. (1990). The fimD gene required for cell surface localization of Escherichia coli type 1 fimbriae. *Mol Gen Genet* **220**, 334-338.

Klionsky, D. J., Abeliovich, H., Agostinis, P., Agrawal, D. K., Aliev, G., Askew, D. S., Baba, M., Baehrecke, E. H., Bahr, B. A., Ballabio, A., Bamber, B. A.,Bassham, D. C., Bergamini, E., Bi, X., Biard-Piechaczyk, M., Blum, J. S., Bredesen, D. E., Brodsky, J. L., Brumell, J. H., Brunk, U. T., Bursch, W., Camougrand, N., Cebollero, E., Cecconi, F., Chen, Y., Chin, L. S., Choi, A., Chu, C. T., Chung, J., Clarke, P. G., Clark, R. S., Clarke, S. G., Clave, C., Cleveland, J. L., Codogno, P., Colombo, M. I., Coto-Montes, A., Cregg, J. M., Cuervo, A. M., Debnath, J., Demarchi, F., Dennis, P. B., Dennis, P. A., Deretic, V., Devenish, R. J., Di Sano, F., Dice, J. F., Difiglia, M., Dinesh-Kumar, S., Distelhorst, C. W., Djavaheri-Mergny, M., Dorsey, F. C., Droge, W., Dron, M., Dunn, W. A., Jr., Duszenko, M., Eissa, N. T., Elazar, Z., Esclatine, A., Eskelinen, E. L., Fesus, L., Finley, K. D., Fuentes, J. M., Fueyo, J., Fujisaki, K., Galliot, B., Gao, F. B., Gewirtz, D. A., Gibson, S. B., Gohla, A., Goldberg, A. L., Gonzalez, R., Gonzalez, Estevez, C., Gorski, S., Gottlieb, R. A., Haussinger, D., He, Y. W., Heidenreich, K., Hill, J. A., Hoyer-Hansen, M., Hu, X., Huang, W. P., Iwasaki, A., Jaattela, M., Jackson, W. T., Jiang, X., Jin, S., Johansen, T., Jung, J. U., Kadowaki, M., Kang, C., Kelekar, A., Kessel, D. H., Kiel, J. A., Kim, H. P., Kimchi, A., Kinsella, T. J., Kiselyov, K.,, Kitamoto, K., Knecht, E., Komatsu, M., Kominami, E., Kondo, S., Kovacs, A. L., Kroemer, G., Kuan, C. Y., Kumar, R., Kundu, M.,

Landry, J., Laporte, M., Le, W., Lei, H. Y., Lenardo, M. J., Levine, B., Lieberman, A., Lim, K. L., Lin, F. C., Liou, W., Liu, L. F., Lopez-Berestein, G., Lopez-Otin, C., Lu, B., Macleod, K. F., Malorni, W., Martinet, W., Matsuoka, K., Mautner, J., Meijer, A. J., Melendez, A., Michels,, P., Miotto, G., Mistiaen, W. P., Mizushima, N., Mograbi, B., Monastyrska, I., Moore, M. N., Moreira, P. I., Moriyasu, Y., Motyl, T., Munz, C., Murphy, L. O., Naqvi, N. I., Neufeld, T. P., Nishino, I., Nixon, R. A., Noda, T., Nurnberg, B., Ogawa, M., Oleinick, N. L., Olsen, L. J., Ozpolat, B., Paglin, S., Palmer, G. E., Papassideri, I., Parkes, M., Perlmutter, D.H., Perry, G., Piacentini, M., Pinkas-Kramarski, R., Prescott, M., Proikas-Cezanne, T., Raben, N., Rami, A., Reggiori, F. Rohrer, B., Rubinsztein, D. C., Ryan, K. M., Sadoshima, J., Sakagami, H., Sakai, Y., Sandri, M., Sasakawa, C., Sass, M., Schneider, C., Seglen, P. O.,, Seleverstov, O., Settleman, J., Shacka, J. J., Shapiro, I. M., Sibirny, A., Silva-Zacarin, E. C., Simon, H. U., Simone, C., Simonsen, A., Smith, M. A., Spanel-Borowski, K., Srinivas, V., Steeves, M., Stenmark, H., Stromhaug, P. E., Subauste, C. S., Sugimoto, S., Sulzer, D., Suzuki, T., Swanson, M. S., Tabas, I., Takeshita, F., Talbot, N. J., Talloczy, Z., Tanaka, K.,, Tanida, I., Taylor, G. S., Taylor, J. P., Terman, A., Tettamanti, G., Thompson, C. B., Thumm, M., Tolkovsky, A. M., Tooze, S. A., Truant, R., Tumanovska, L. V., Uchiyama, Y., Ueno, T., Uzcategui, N. L., van der Klei, I., Vaquero, E. C., Vellai, T., Vogel, M. W., Wang, H. G., Webster, P., Wiley, J. W., Xi, Z., Xiao, G., Yahalom, J., Yang, J. M.
Yap, G., Yin, X. M., Yoshimori, T., Yu, L., Yue, Z., Yuzaki, M., Zabirnyk, O., Zheng, X., Zhu, X. & Deter, R. L. (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**, 151-175.

Knight, P., Campbell, B. J. & Rhodes, J. M. (2008). Host-bacteria interaction in inflammatory bowel disease. *Br Med Bull* **88**, 95-113.

Kobayashi, K., Inohara, N., Hernandez, L. D., Galan, J. E., Nunez, G., Janeway, C. A., Medzhitov, R. & Flavell, R. A. (2002). RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* **416**, 194-199.

Korhonen, T. K., Virkola, R. & Holthofer, H. (1986). Localization of binding sites for purified Escherichia coli P fimbriae in the human kidney. *Infect Immun* **54**, 328-332.

Kotlowski, R., Bernstein, C. N., Sepehri, S. & Krause, D. O. (2007). High prevalence of Escherichia coli belonging to the B2+D phylogenetic group in inflammatory bowel disease. *Gut* **56**, 669-675.

Kraehenbuhl, J. P. & Neutra, M. R. (2000). Epithelial M cells: differentiation and function. *Annu Rev Cell Dev Biol* **16**, 301-332.

Krause, D. O., Little, A. C., Dowd, S. E. & Bernstein, C. N. (2010). Complete genome sequence of adherent invasive Escherichia coli UM146 isolated from Ileal Crohn's disease biopsy tissue. *J Bacteriol* **193**, 583.

Kuballa, P., Huett, A., Rioux, J. D., Daly, M. J. & Xavier, R. J. (2008). Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS ONE* **3**, e3391.

Kucheria, R., Dasgupta, P., Sacks, S. H., Khan, M. S. & Sheerin, N. S. (2005). Urinary tract infections: new insights into a common problem. *Postgrad Med J* **81**, 83-86.

Kuehn, M. J. & Kesty, N. C. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev* **19**, 2645-2655.

Kurthkoti, K., Kumar, P., Jain, R. & Varshney, U. (2008). Important role of the nucleotide excision repair pathway in Mycobacterium smegmatis in conferring protection against commonly encountered DNA-damaging agents. *Microbiology* **154**, 2776-2785.

Kurthkoti, K. & Varshney, U. (2011). Distinct mechanisms of DNA repair in mycobacteria and their implications in attenuation of the pathogen growth. *Mech Ageing Dev* **133**, 138-146.

La Scola, B. & Raoult, D. (2001). Survival of Coxiella burnetii within free-living amoeba Acanthamoeba castellanii. *Clin Microbiol Infect* **7**, 75-79.

Laffineur, G., Lescut, D., Vincent, P., Quandalle, P., Wurtz, A. & Colombel, J. F. (1992). [Bacterial translocation in Crohn disease]. *Gastroenterol Clin Biol* **16**, 777-781.

Lahiri, R. & Krahenbuhl, J. L. (2008). The role of free-living pathogenic amoeba in the transmission of leprosy: a proof of principle. *Lepr Rev* **79**, 401-409.

Lala, S., Ogura, Y., Osborne, C., Hor, S. Y., Bromfield, A., Davies, S., Ogunbiyi, O., Nunez, G. & Keshav, S. (2003). Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* **125**, 47-57.

Lamarche, M. G., Dozois, C. M., Daigle, F., Caza, M., Curtiss, R., 3rd, Dubreuil, J. D. & Harel, J. (2005). Inactivation of the pst system reduces the virulence of an avian pathogenic Escherichia coli O78 strain. *Infect Immun* **73**, 4138-4145.

Lamarche, M. G., Wanner, B. L., Crepin, S. & Harel, J. (2008). The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiol Rev* **32**, 461-473.

Lamers, M. H., Perrakis, A., Enzlin, J. H., Winterwerp, H. H., de Wind, N. & Sixma, T. K. (2000). The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. *Nature* **407**, 711-717.

Landers, C. J., Cohavy, O., Misra, R., Yang, H., Lin, Y. C., Braun, J. & Targan, S. R. (2002). Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. *Gastroenterology* **123**, 689-699.

Lapaquette, P., Bringer, M. A. & Darfeuille-Michaud, A. (2012a). Defects in autophagy favour adherent-invasive Escherichia coli persistence within macrophages leading to increased pro-inflammatory response. *Cell Microbiol*.

Lapaquette, P., Bringer, M. A. & Darfeuille-Michaud, A. (2012b). Defects in autophagy favour adherent-invasive Escherichia coli persistence within macrophages leading to increased pro-inflammatory response. *Cell Microbiol* **14**, 791-807.

Lapaquette, P., Glasser, A. L., Huett, A., Xavier, R. J. & Darfeuille-Michaud, A. (2012c). Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol* **12**, 99-113.

Laub, M. T. & Goulian, M. (2007). Specificity in two-component signal transduction pathways. *Annu Rev Genet* **41**, 121-145.

Lecine, P., Esmiol, S., Metais, J. Y. & other authors (2007). The NOD2-RICK complex signals from the plasma membrane. *J Biol Chem* **282**, 15197-15207.

Lee, K. Y., Yu, C. S., Cho, Y. B., Park, K. J., Choi, G. S., Yoon, S. N. & Yoo, H. (2012). Risk factors for repeat abdominal surgery in korean patients with Crohn's disease: a multi-center study of a korean inflammatory bowel disease study group. *J Korean Soc Coloproctol* **28**, 188-194.

Lee, S., Yu, J. K., Park, K., Oh, E. J., Kim, S. Y. & Park, Y. J. (2010). Phylogenetic groups and virulence factors in pathogenic and commensal strains of Escherichia coli and their association with blaCTX-M. *Ann Clin Lab Sci* **40**, 361-367.

Lesage, S., Zouali, H., Cezard, J. P., Colombel, J. F., Belaiche, J., Almer, S., Tysk, C., O'Morain, C., Gassull, M., Binder, V., Finkel, Y., Modigliani, R., Gower-Rousseau, C., Macry, J., Merlin, F., Chamaillard, M., Jannot, A. S., Thomas, G., Hugot, J. P. (2002). CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* **70**, 845-857.

Leuko, S. & Raivio, T. L. (2012). Mutations that impact the enteropathogenic Escherichia coli (EPEC) Cpx envelope stress response attenuate virulence in Galleria mellonella. *Infect Immun*.

Levin, J. D., Johnson, A. W. & Demple, B. (1988). Homogeneous Escherichia coli endonuclease IV. Characterization of an enzyme that recognizes oxidative damage in DNA. *J Biol Chem* **263**, 8066-8071.

Li, Q., Wang, C., Tang, C., Li, N. & Li, J. (2012). Molecular-phylogenetic characterization of the microbiota in ulcerated and non-ulcerated regions in the patients with Crohn's disease. *PLoS ONE* **7**, e34939.

Libioulle, C., Louis, E., Hansoul, S. & other authors (2007). Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genet* **3**, e58.

Lindstedt, B. A., Brandal, L. T., Aas, L., Vardund, T. & Kapperud, G. (2007). Study of polymorphic variable-number of tandem repeats loci in the ECOR collection and in a set of pathogenic Escherichia coli and Shigella isolates for use in a genotyping assay. *J Microbiol Methods* **69**, 197-205.

Linn, S. & Imlay, J. A. (1987). Toxicity, mutagenesis and stress responses induced in Escherichia coli by hydrogen peroxide. *J Cell Sci Suppl* **6**, 289-301.

Liochev, S. I. & Fridovich, I. (1992). Fumarase C, the stable fumarase of Escherichia coli, is controlled by the soxRS regulon. *Proc Natl Acad Sci U S A* **89**, 5892-5896.

Lippa, A. M. & Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* **5**, e1000788.

Liu, J., Obi, I. R., Thanikkal, E. J., Kieselbach, T. & Francis, M. S. (2011). Phosphorylated CpxR restricts production of the RovA global regulator in Yersinia pseudotuberculosis. *PLoS ONE* **6**, e23314.

Liu, Y., van Kruiningen, H. J., West, A. B., Cartun, R. W., Cortot, A. & Colombel, J. F. (1995). Immunocytochemical evidence of Listeria, Escherichia coli, and Streptococcus antigens in Crohn's disease. *Gastroenterology* **108**, 1396-1404.

Llewellyn, A. C., Jones, C. L., Napier, B. A., Bina, J. E. & Weiss, D. S. (2011). Macrophage replication screen identifies a novel Francisella hydroperoxide resistance protein involved in virulence. *PLoS ONE* **6**, e24201.

Lodes, M. J., Cong, Y., Elson, C. O., Mohamath, R., Landers, C. J., Targan, S. R., Fort, M. & Hershberg, R. M. (2004). Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* **113**, 1296-1306.

Lodes, M. J., Y. Cong, C. O. Elson, R. Mohamath, C. J. Landers, S. R.Targan, M. Fort, and R. M. Hershberg. (2004). Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Investig* **113:**, 1296–1306.

Loewen, P. C., Triggs, B. L., George, C. S. & Hrabarchuk, B. E. (1985). Genetic mapping of katG, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in Escherichia coli. *J Bacteriol* **162**, 661-667.

Loewen, P. C., Switala, J., von Ossowski, I., Hillar, A., Christie, A., Tattrie, B. & Nicholls, P. (1993). Catalase HPII of Escherichia coli catalyzes the conversion of protoheme to cis-heme d. *Biochemistry* **32**, 10159-10164.

Loftus Jr., E. V. (2004). Clinical epidemiology of inflammatory bowel disease: incidence, prevalence and environmental influences. *Gastroenterology* **126:**, 1504-1517.

Lombard, D. B., Chua, K. F., Mostoslavsky, R., Franco, S., Gostissa, M. & Alt, F. W. (2005). DNA repair, genome stability, and aging. *Cell* **120**, 497-512.

Louis, E., Michel, V., Hugot, J. P., Reenaers, C., Fontaine, F., Delforge, M., El Yafi, F., Colombel, J. F. & Belaiche, J. (2003). Early development of stricturing or penetrating pattern in Crohn's disease is influenced by disease location, number of flares, and smoking but not by NOD2/CARD15 genotype. *Gut* **52**, 552-557.

Louis, P., McCrae, S.I., Charrier, C. and Flint, H.J. (2007). Organization of butyrate synthetic genes in human colonic bacteria: phylogenetic conservation and horizontal gene transfer. *FEMS Microbiol* **269**, 240-247.

Lu, A. L., Li, X., Gu, Y., Wright, P. M. & Chang, D. Y. (2001). Repair of oxidative DNA damage: mechanisms and functions. *Cell Biochem Biophys* **35**, 141-170.

Lukjancenko, O., Wassenaar, T. M. & Ussery, D. W. (2010). Comparison of 61 sequenced Escherichia coli genomes. *Microb Ecol* **60**, 708-720.

Ly, T. M. & Muller, H. E. (1990). Ingested Listeria monocytogenes survive and multiply in protozoa. *J Med Microbiol* **33**, 51-54.

Ma, Z., Masuda, N. & Foster, J. W. (2004). Characterization of EvgAS-YdeO-GadE branched regulatory circuit governing glutamate-dependent acid resistance in Escherichia coli. *J Bacteriol* **186**, 7378-7389.

Macpherson, A., Khoo, U. Y., Forgacs, I., Philpott-Howard, J. & Bjarnason, I. (1996). Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* **38**, 365-375.

Macritchie, D. M., Ward, J. D., Nevesinjac, A. Z. & Raivio, T. L. (2008). Activation of the Cpx envelope stress response down-regulates expression of several locus of enterocyte effacement-encoded genes in enteropathogenic Escherichia coli. *Infect Immun* **76**, 1465-1475.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140-3145.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E. & other authors (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* **55**, 205-211.

Marquart, M. E., Picking, W. L. & Picking, W. D. (1996). Soluble invasion plasmid antigen C (IpaC) from Shigella flexneri elicits epithelial cell responses related to pathogen invasion. *Infect Immun* **64**, 4182-4187.

Marra, A. & Isberg, R. R. (1997). Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium. *Infect Immun* **65**, 3412-3421.

Martin, H. M., Campbell, B. J., Hart, C. A., Mpofu, C., Nayar, M., Singh, R., Englyst, H., Williams, H. F. & Rhodes, J. M. (2004). Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology* **127**, 80-93.

Martin, H. M., Campbell, B.J., Hart, C.A., Mpofu, C., Nayar, M., Singh, R., Englyst, H., Williams, H.F., and Rhodes, J.M. (2004). Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. . *Gastroenterology* **127:**, 80-93.

Martinez-Argudo, I., Sands, C. & Jepson, M. A. (2007). Translocation of enteropathogenic Escherichia coli across an in vitro M cell model is regulated by its type III secretion system. *Cell Microbiol* **9**, 1538-1546.

Martinez-Medina, M., Aldeguer, X., Lopez-Siles, M., Gonzalez-Huix, F., Lopez-Oliu, C., Dahbi, G., Blanco, J. E., Blanco, J., Garcia-Gil, L. J. & Darfeuille-Michaud, A. (2009a). Molecular diversity of Escherichia coli in the human gut: new ecological evidence supporting the role of adherent-invasive E. coli (AIEC) in Crohn's disease. *Inflamm Bowel Dis* **15**, 872-882.

Martinez-Medina, M., Aldeguer, X., Lopez-Siles, Gonzalez-Huix, F., Lopez-Oliu, C., Dahbi, G., Blanco, J. E., Blanco, J., Garcia-Gil, L. J. & Darfeuille-Michaud, A. (2009b). Molecular diversity of *Escherichia coli* in the human gut: new ecological evidence supporting the role of adherent-invasive *E. coli* (AIEC) in Crohn's disease. *Inflamm Bowel Dis* **15**, 872-882.

Martinez-Medina, M., Mora, A., Blanco, M., Lopez, C., Alonso, M. P., Bonacorsi, S., Nicolas-Chanoine, M. H., Darfeuille-Michaud, A., Garcia-Gil, J. & Blanco, J. (2009c). Similarity and divergence among adherent-invasive Escherichia coli and extraintestinal pathogenic E. coli strains. *J Clin Microbiol* **47**, 3968-3979.

Martinez-Medina, M., Mora, A., Blanco, Lopez, C., Alonso, M. P., Bonacorsi, S., Nicolas-Chanoine, M. H., Darfeuille-Michaud, A., Garcia-Gil, J. & Blanco, J. (2009d). Similarity and Divergence among Adherent-Invasive *Escherichia coli* and Extraintestinal Pathogenic *E. coli* Strains. *J Clin Microbiol* **47**, 3968-3979.

Martinez-Medina, M., Garcia-Gil, J., Barnich, N., Wieler, L. H. & Ewers, C. (2011). Adherent-invasive Escherichia coli phenotype displayed by intestinal pathogenic E. coli strains from cats, dogs, and swine. *Appl Environ Microbiol* **77**, 5813-5817.

Mates, A. K., Sayed, A. K. & Foster, J. W. (2007). Products of the Escherichia coli acid fitness island attenuate metabolite stress at extremely low pH and mediate a cell density-dependent acid resistance. *J Bacteriol* **189**, 2759-2768.

Matz, C., Bergfeld, T., Rice, S. A. & Kjelleberg, S. (2004). Microcolonies, quorum sensing and cytotoxicity determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing. *Environ Microbiol* **6**, 218-226.

Matz, C. & Kjelleberg, S. (2005). Off the hook--how bacteria survive protozoan grazing. *Trends Microbiol* **13**, 302-307.

Mau, B., Glasner, J. D., Darling, A. E. & Perna, N. T. (2006). Genome-wide detection and analysis of homologous recombination among sequenced strains of Escherichia coli. *Genome Biol* **7**, R44.

McCarroll, S. A., Huett, A., Kuballa, P. & other authors (2008). Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat Genet* **40**, 1107-1112.

McClain, M. S., Blomfield, I. C. & Eisenstein, B. I. (1991). Roles of fimB and fimE in site-specific DNA inversion associated with phase variation of type 1 fimbriae in Escherichia coli. *J Bacteriol* **173**, 5308-5314.

McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S. & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci U S A* **92**, 1664-1668.

Meconi, S., Vercellone, A., Levillain, F., Payre, B., Al Saati, T., Capilla, F., Desreumaux, P., Darfeuille-Michaud, A. & Altare, F. (2007). Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol* **9**, 1252-1261.

Meconi, S., Vercellone, A., Levillain, F., Payre, B., Al Saati, T., Capilla, F., Desreumaux, P., Darfeuille-Michaud, A., and Altare, F. (2007). Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol* **9:**, 1252-1261.

Mecsas, J., Rouviere, P. E., Erickson, J. W., Donohue, T. J. & Gross, C. A. (1993). The activity of sigma E, an Escherichia coli heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev* **7**, 2618-2628.

Meduri, G. U., Kanangat, S., Bronze, M., Patterson, D. R., Meduri, C. U., Pak, C., Tolley, E. A. & Schaberg, D. R. (2001). Effects of methylprednisolone on intracellular bacterial growth. *Clin Diagn Lab Immunol* **8**, 1156-1163.

Mei, L., Targan, S. R., Landers, C. J., Dutridge, D., Ippoliti, A., Vasiliauskas, E. A., Papadakis, K. A., Fleshner, P. R.,Rotter, J. I. & Yang, H. (2006). Familial expression of anti-Escherichia coli outer membrane porin C in relatives of patients with Crohn's disease. *Gastroenterology* **130**, 1078-1085.

Mellmann, A., Harmsen, D., Cummings, C. A. & other authors (2011). Prospective genomic characterization of the German enterohemorrhagic Escherichia coli O104:H4 outbreak by rapid next generation sequencing technology. *PLoS ONE* **6**, e22751.

Mertens, K., Lantsheer, L. & Samuel, J. E. (2005). A minimal set of DNA repair genes is sufficient for survival of Coxiella burnetii under oxidative stress. *Ann N Y Acad Sci* **1063**, 73-75.

Milkman, R. (1973). Electrophoretic variation in Escherichia coli from natural sources. *Science* **182**, 1024-1026.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T. & Hermon-Taylor, J. (1996). IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurized cows' milk in England and Wales. *Appl Environ Microbiol* **62**, 3446-3452.

Miltner, E. C. & Bermudez, L. E. (2000). Mycobacterium avium grown in Acanthamoeba castellanii is protected from the effects of antimicrobials. *Antimicrob Agents Chemother* **44**, 1990-1994.

Minamino, T. & MacNab, R. M. (2000). FliH, a soluble component of the type III flagellar export apparatus of Salmonella, forms a complex with FliI and inhibits its ATPase activity. *Mol Microbiol* **37**, 1494-1503.

Miquel, S., Peyretaillade, E., Claret, L., de Vallee, A., Dossat, C., Vacherie, B., Zineb el, H., Segurens, B., Barbe, V., Sauvanet, P., Neut, C., Colombel, J. F., Medigue, C., Mojica, F. J., Peyret, P., Bonnet, R., Darfeuille-Michaud, A. (2010). Complete genome sequence of Crohn's disease-associated adherent-invasive E. coli strain LF82. *PLoS ONE* **5**.

Mitobe, J., Arakawa, E. & Watanabe, H. (2005). A sensor of the two-component system CpxA affects expression of the type III secretion system through posttranscriptional processing of InvE. *J Bacteriol* **187**, 107-113.

Mizuno, T. (1997). Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of Escherichia coli. *DNA Res* **4**, 161-168.

Modrich, P. (1994). Mismatch repair, genetic stability, and cancer. *Science* **266**, 1959-1960.

Mondot, S., Kang, S., Furet, J. P., Aguirre de Carcer, D., McSweeney, C., Morrison, M., Marteau, P., Dore, J. & Leclerc, M. (2011). Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm Bowel Dis* **17**, 185-192.

Monsieurs, P., De Keersmaecker, S., Navarre, W. W., Bader, M. W., De Smet, F., McClelland, M., Fang, F. C., De Moor, B., Vanderleyden, J. & Marchal, K. (2005). Comparison of the PhoPQ regulon in Escherichia coli and Salmonella typhimurium. *J Mol Evol* **60**, 462-474.

Moon, H. W., Whipp, S. C., Argenzio, R. A., Levine, M. M. & Giannella, R. A. (1983). Attaching and effacing activities of rabbit and human enteropathogenic Escherichia coli in pig and rabbit intestines. *Infect Immun* **41**, 1340-1351.

Moon, K. & Gottesman, S. (2009). A PhoQ/P-regulated small RNA regulates sensitivity of Escherichia coli to antimicrobial peptides. *Mol Microbiol* **74**, 1314-1330.

Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G. & Ames, B. N. (1986). Hydrogen peroxide-inducible proteins in Salmonella typhimurium overlap with heat shock and other stress proteins. *Proc Natl Acad Sci U S A* **83**, 8059-8063.

Motta, E. S., Souza-Santos, P. T., Cassiano, T. R., Dantas, F. J., Caldeira-de-Araujo, A. & De Mattos, J. C. (2010). Endonuclease IV is the main base excision repair enzyme involved in DNA damage induced by UVA radiation and stannous chloride. *J Biomed Biotechnol* **2010**, 376218.

Mow, W. S., Landers, C. J., Steinhart, A. H., Feagan, B. G., Croitoru, K., Seidman, E., Greenberg, G. R. & Targan, S. R. (2004). High-level serum antibodies to bacterial antigens are associated with antibiotic-induced clinical remission in Crohn's disease: a pilot study. *Dig Dis Sci* **49**, 1280-1286.

Mpofu, C. M., Campbell, B. J., Subramanian, S. & other authors (2007). Microbial mannan inhibits bacterial killing by macrophages: a possible pathogenic mechanism for Crohn's disease. *Gastroenterology* **133**, 1487-1498.

Mukhopadhyay, S. & Schellhorn, H. E. (1997). Identification and characterization of hydrogen peroxide-sensitive mutants of Escherichia coli: genes that require OxyR for expression. *J Bacteriol* **179**, 330-338.

Mulvey, M. A., Lopez-Boado, Y. S., Wilson, C. L., Roth, R., Parks, W. C., Heuser, J. & Hultgren, S. J. (1998). Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. *Science* **282**, 1494-1497.

Mulvey, M. A., Schilling, J. D. & Hultgren, S. J. (2001). Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. *Infect Immun* **69**, 4572-4579.

Mulvey, M. A. (2002). Adhesion and entry of uropathogenic Escherichia coli. *Cell Microbiol* **4**, 257-271.

Mylonaki, M., Rayment, N. B., Rampton, D. S., Hudspith, B. N. & Brostoff, J. (2005). Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* **11**, 481-487.

Nada, R. A., Shaheen, H. I., Khalil, S. B., Mansour, A., El-Sayed, N., Touni, I., Weiner, M., Armstrong, A. W. & Klena, J. D. (2011). Discovery and phylogenetic analysis of novel members of class b enterotoxigenic Escherichia coli adhesive fimbriae. *J Clin Microbiol* **49**, 1403-1410.

Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S. & Yoshimori, T. (2004). Autophagy defends cells against invading group A Streptococcus. *Science* **306**, 1037-1040.

Naser, S. A., Ghobrial, G., Romero, C. & Valentine, J. F. (2004). Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* **364**, 1039-1044.

Naser, S. A., Arce, M., Khaja, A., Fernandez, M., Naser, N., Elwasila, S. & Thanigachalam, S. (2012). Role of ATG16L, NOD2 and IL23R in Crohn's disease pathogenesis. *World J Gastroenterol* **18**, 412-424.

Nash, J. H., Villegas, A., Kropinski, A. M., Aguilar-Valenzuela, R., Konczy, P., Mascarenhas, M., Ziebell, K., Torres, A. G., Karmali, M. A., Coombes, B. K. (2010). Genome sequence of adherent-invasive Escherichia coli and comparative genomic analysis with other E. coli pathotypes. *BMC Genomics* **11**, 667.

Nataro, J. P., Kaper, J. B., Robins-Browne, R., Prado, V., Vial, P. & Levine, M. M. (1987). Patterns of adherence of diarrheagenic Escherichia coli to HEp-2 cells. *Pediatr Infect Dis J* **6**, 829-831.

Nataro, J. P., Deng, Y., Maneval, D. R., German, A. L., Martin, W. C. & Levine, M. M. (1992). Aggregative adherence fimbriae I of enteroaggregative Escherichia coli mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infect Immun* **60**, 2297-2304.

Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic Escherichia coli. *Clin Microbiol Rev* **11**, 142-201.

Nataro, J. P., Steiner, T. & Guerrant, R. L. (1998). Enteroaggregative Escherichia coli. *Emerg Infect Dis* **4**, 251-261.

Nathan, C. & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* **97**, 8841-8848.

Navarro-Garcia, F., Eslava, C., Villaseca, J. M., Lopez-Revilla, R., Czeczulin, J. R., Srinivas, S., Nataro, J. P. & Cravioto, A. (1998). In vitro effects of a high-molecular-weight heat-labile enterotoxin from enteroaggregative Escherichia coli. *Infect Immun* **66**, 3149-3154.

Neut, C., Bulois, P., Desreumaux, P., Membre, J. M., Lederman, E., Gambiez, L., Cortot, A., Quandalle, P., van Kruiningen, H. & Colombel, J. F. (2002). Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *Am J Gastroenterol* **97**, 939-946.

Neutra, M. R., Mantis, N. J., Frey, A. & Giannasca, P. J. (1999). The composition and function of M cell apical membranes: implications for microbial pathogenesis. *Semin Immunol* **11**, 171-181.

Nevesinjac, A. Z. & Raivio, T. L. (2005). The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic Escherichia coli. *J Bacteriol* **187**, 672-686.

Niederhoffer, E. C., Naranjo, C. M., Bradley, K. L. & Fee, J. A. (1990). Control of Escherichia coli superoxide dismutase (sodA and sodB) genes by the ferric uptake regulation (fur) locus. *J Bacteriol* **172**, 1930-1938.

Noriega, F. R., Liao, F. M., Formal, S. B., Fasano, A. & Levine, M. M. (1995). Prevalence of Shigella enterotoxin 1 among Shigella clinical isolates of diverse serotypes. *J Infect Dis* **172**, 1408-1410.

Nos, P. & Domenech, E. (2011). Management of Crohn's disease in smokers: is an alternative approach necessary? *World J Gastroenterol* **17**, 3567-3574.

O'Brien, A. D., Newland, J. W., Miller, S. F., Holmes, R. K., Smith, H. W. & Formal, S. B. (1984). Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. *Science* **226**, 694-696.

O'Rourke, E. J., Chevalier, C., Pinto, A. V., Thiberge, J. M., Ielpi, L., Labigne, A. & Radicella, J. P. (2003). Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in Helicobacter pylori colonization. *Proc Natl Acad Sci U S A* **100**, 2789-2794.

Ochman, H., Whittam, T. S., Caugant, D. A. & Selander, R. K. (1983). Enzyme polymorphism and genetic population structure in Escherichia coli and Shigella. *J Gen Microbiol* **129**, 2715-2726.

Ochman, H. & Selander, R. K. (1984a). Standard reference strains of Escherichia coli from natural populations. *J Bacteriol* **157**, 690-693.

Ochman, H. & Selander, R. K. (1984b). Evidence for clonal population structure in Escherichia coli. *Proc Natl Acad Sci U S A* **81**, 198-201.

Ochman, H., Lawrence, J. G. & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299-304.

Ogura, Y., Bonen, D. K., Inohara, N. & other authors (2001a). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603-606.

Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S. & Nunez, G. (2001b). Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* **276**, 4812-4818.

Ohnishi, K., Ohto, Y., Aizawa, S., Macnab, R. M. & Iino, T. (1994). FlgD is a scaffolding protein needed for flagellar hook assembly in Salmonella typhimurium. *J Bacteriol* **176**, 2272-2281.

Okamoto, K., Yamanaka, H., Takeji, M. & Fuji, Y. (2001). Region of heat-stable enterotoxin II of Escherichia coli involved in translocation across the outer membrane. *Microbiol Immunol* **45**, 349-355.

Otto, K. & Silhavy, T. J. (2002). Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci U S A* **99**, 2287-2292.

Palomino-Morales, R. J., Oliver, J., Gomez-Garcia, M., Lopez-Nevot, M. A., Rodrigo, L., Nieto, A., Alizadeh, B. Z. & Martin, J. (2009). Association of ATG16L1 and IRGM genes polymorphisms with inflammatory bowel disease: a meta-analysis approach. *Genes Immun* **10**, 356-364.

Parkes, M., Barrett, J. C., Prescott, N. J. & other authors (2007). Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet* **39**, 830-832.

Parkkinen, J., Korhonen, T. K., Pere, A., Hacker, J. & Soinila, S. (1988). Binding sites in the rat brain for Escherichia coli S fimbriae associated with neonatal meningitis. *J Clin Invest* **81**, 860-865.

Pedersen, L. L., Radulic, M., Doric, M. & Abu Kwaik, Y. (2001). HtrA Homologue of Legionella pneumophila: an Indispensable Element for Intracellular Infection of Mammalian but Not Protozoan Cells. *Infect Immun* **69**, 2569-2579.

Peiffer, I., Servin, A. L. & Bernet-Camard, M. F. (1998). Piracy of decay-accelerating factor (CD55) signal transduction by the diffusely adhering strain Escherichia coli C1845 promotes cytoskeletal F-actin rearrangements in cultured human intestinal INT407 cells. *Infect Immun* **66**, 4036-4042.

Peltekova, V. D., Wintle, R. F., Rubin, L. A., Amos, C. I., Huang, Q., Gu, X., Newman, B., Van Oene, M., Cescon, D., Greenberg, G., Griffiths, A. M., St George-Hyslop, P. H., & Siminovitch, K. A. (2004). Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet* **36**, 471-475.

Perna, N. T., Plunkett, G., 3rd, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G.F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K.D. Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A. & Blattner, F. R. (2001). Genome sequence of enterohaemorrhagic Escherichia coli O157:H7. *Nature* **409**, 529-533.

Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**, 785-786.

Pidasheva, S., Trifari, S., Phillips, A., Hackney, J. A., Ma, Y., Smith, A., Sohn, S. J., Spits, H., Little, R. D., Behrens, T. W., Honigberg, L., Ghilardi, N. & Clark, H. F. (2011). Functional studies on the IBD susceptibility gene IL23R implicate reduced receptor function in the protective genetic variant R381Q. *PLoS ONE* **6**, e25038.

Plantinga, T. S., Joosten, L. A., van der Meer, J. W. & Netea, M. G. (2012). Modulation of inflammation by autophagy: consequences for Crohn's disease. *Curr Opin Pharmacol*.

Pomposiello, P. J., Koutsolioutsou, A., Carrasco, D. & Demple, B. (2003). SoxRS-regulated expression and genetic analysis of the yggX gene of Escherichia coli. *J Bacteriol* **185**, 6624-6632.

Porat, R., Clark, B. D., Wolff, S. M. & Dinarello, C. A. (1991). Enhancement of growth of virulent strains of Escherichia coli by interleukin-1. *Science* **254**, 430-432.

Prasadarao, N. V., Wass, C. A. & Kim, K. S. (1996). Endothelial cell GlcNAc beta 1-4GlcNAc epitopes for outer membrane protein A enhance traversal of Escherichia coli across the blood-brain barrier. *Infect Immun* **64**, 154-160.

Prasadarao, N. V. (2002). Identification of Escherichia coli outer membrane protein A receptor on human brain microvascular endothelial cells. *Infect Immun* **70**, 4556-4563.

Prescott, N. J., Fisher, S. A., Franke, A. & other authors (2007). A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5. *Gastroenterology* **132**, 1665-1671.

Price, N. L. & Raivio, T. L. (2009). Characterization of the Cpx regulon in Escherichia coli strain MC4100. *J Bacteriol* **191**, 1798-1815.

Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P. & Dorel, C. (2001). Complex regulatory network controls initial adhesion and biofilm formation in Escherichia coli via regulation of the csgD gene. *J Bacteriol* **183**, 7213-7223.

Pullan, R. D., Thomas, G. A., Rhodes, M., Newcombe, R. G., Williams, G. T., Allen, A. & Rhodes, J. (1994). Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut* **35**, 353-359.

Pupo, G. M. & Richardson, B. J. (1995). Biochemical genetics of a natural population of Escherichia coli: seasonal changes in alleles and haplotypes. *Microbiology* **141 (Pt 4)**, 1037-1044.

Puspok, A., Dejaco, C., Oberhuber, G., Waldhor, T., Hirschl, A. M., Vogelsang, H. & Gasche, C. (1999). Influence of Helicobacter pylori infection on the phenotype of Crohn's disease. *Am J Gastroenterol* **94**, 3239-3244.

Raivio, T. L., Popkin, D. L. & Silhavy, T. J. (1999). The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J Bacteriol* **181**, 5263-5272.

Raivio, T. L., Laird, M. W., Joly, J. C. & Silhavy, T. J. (2000). Tethering of CpxP to the inner membrane prevents spheroplast induction of the cpx envelope stress response. *Mol Microbiol* **37**, 1186-1197.

Raivio, T. L. (2005). Envelope stress responses and Gram-negative bacterial pathogenesis. *Mol Microbiol* **56**, 1119-1128.

Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K. & Whittam, T. S. (2000). Parallel evolution of virulence in pathogenic Escherichia coli. *Nature* **406**, 64-67.

Rioux, J. D., Daly, M. J., Silverberg, M. S. & other authors (2001). Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat Genet* **29**, 223-228.

Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M. M., Datta, L. W., Shugart, Y. Y., Griffiths, A. M., Targan, S. R., Ippoliti, A. F., Bernard, E. J., Mei, L., Nicolae, D. L., Regueiro, M., Schumm, L. P., Steinhart, A. H., Rotter, J. I., Duerr, R. H., Cho, J. H., Daly, M. J. & Brant, S. R. (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* **39**, 596-604.

Roberts, C. L., Keita, A. V., Duncan, S. H., O'Kennedy, N., Soderholm, J. D., Rhodes, J. M. & Campbell, B. J. (2010). Translocation of Crohn's disease Escherichia coli across M-cells: contrasting effects of soluble plant fibres and emulsifiers. *Gut* **59**, 1331-1339.

Robinson, J. M. (2008). Reactive oxygen species in phagocytic leukocytes. *Histochem Cell Biol* **130**, 281-297.

Rolhion, N., Barnich, N., Claret, L. & Darfeuille-Michaud, A. (2005). Strong decrease in invasive ability and outer membrane vesicle release in Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 with the yfgL gene deleted. *J Bacteriol* **187**, 2286-2296.

Rolhion, N., Carvalho, F. A. & Darfeuille-Michaud, A. (2007). OmpC and the sigma(E) regulatory pathway are involved in adhesion and invasion of the Crohn's disease-associated Escherichia coli strain LF82. *Mol Microbiol* **63**, 1684-1700.

Rolhion, N., Barnich, N., Bringer, M. A., Glasser, A. L., Ranc, J., Hebuterne, X., Hofman, P. & Darfeuille-Michaud, A. (2010). Abnormally expressed ER stress response chaperone Gp96 in CD favours adherent-invasive Escherichia coli invasion. *Gut* **59**, 1355-1362.

Rolhion, N., Hofman, P. & Darfeuille-Michaud, A. (2011). The endoplasmic reticulum stress response chaperone: Gp96, a host receptor for Crohn disease-associated adherent-invasive Escherichia coli. *Gut Microbes* **2**, 115-119.

Rolhion, N., and Darfeuille-Michaud, A. (2007). Adherent-invasive Escherichia coli in inflammatory bowel disease. *Inflamm Bowel Dis* **13:**, 1277-1283.

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. *J Clin Pathol* **33**, 1179-1183.

Rowbotham, T. J. (1986). Current views on the relationships between amoebae, legionellae and man. *Isr J Med Sci* **22**, 678-689.

Ryan, P., Kelly, R. G., Lee, G., Collins, J. K., O'Sullivan, G. C., O'Connell, J. & Shanahan, F. (2004). Bacterial DNA within granulomas of patients with Crohn's disease--detection by laser capture microdissection and PCR. *Am J Gastroenterol* **99**, 1539-1543.

Saijo-Hamano, Y., Uchida, N., Namba, K. & Oosawa, K. (2004). In vitro characterization of FlgB, FlgC, FlgF, FlgG, and FliE, flagellar basal body proteins of Salmonella. *J Mol Biol* **339**, 423-435.

Saito, M., Katsuno, T., Nakagawa, T., Sato, T., Noguchi, Y., Sazuka, S., Saito, K., Arai, M. Yokote, K., Yokosuka, O. (2012). Intestinal Epithelial Cells with Impaired Autophagy Lose Their Adhesive Capacity in the Presence of TNF-alpha. *Dig Dis Sci* **8**, 2022-2030.

Saitoh, T., Fujita, N., Jang, M. H., Uematsu, S., Yang, B. G., Satoh, T., Omori, H. Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O., Yoshimori, T., Akira, S. (2008). Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* **456**, 264-268.

Salah, I. B., Ghigo, E. & Drancourt, M. (2009). Free-living amoebae, a training field for macrophage resistance of mycobacteria. *Clin Microbiol Infect* **15**, 894-905.

Sanger, J. M., Chang, R., Ashton, F., Kaper, J. B. & Sanger, J. W. (1996). Novel form of actin-based motility transports bacteria on the surfaces of infected cells. *Cell Motil Cytoskeleton* **34**, 279-287.

Sansonetti, P. (2002). Host-pathogen interactions: the seduction of molecular cross talk. *Gut* **50 Suppl 3**, III2-8.

Sansonetti, P. J. & Phalipon, A. (1999). M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process. *Semin Immunol* **11**, 193-203.

Sansonetti, P. J., Phalipon, A., Arondel, J., Thirumalai, K., Banerjee, S., Akira, S., Takeda, K. & Zychlinsky, A. (2000). Caspase-1 activation of IL-1beta and IL-18 are essential for Shigella flexneri-induced inflammation. *Immunity* **12**, 581-590.

Sartor, R. B. (2005). Does Mycobacterium avium subspecies paratuberculosis cause Crohn's disease? *Gut* **54**, 896-898.

Sartor, R. B. (2006). Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* **3:**, 390-407.

Sartor, R. B. (2009). Microbial-host interactions in inflammatory bowel diseases and experimental colitis. *Nestle Nutr Workshop Ser Pediatr Program* **64**, 121-132; discussion 132-127, 251-127.

Sauer, F. G., Mulvey, M. A., Schilling, J. D., Martinez, J. J. & Hultgren, S. J. (2000). Bacterial pili: molecular mechanisms of pathogenesis. *Curr Opin Microbiol* **3**, 65-72.

Scaletsky, I. C., Fabbricotti, S. H., Carvalho, R. L., Nunes, C. R., Maranhao, H. S., Morais, M. B. & Fagundes-Neto, U. (2002). Diffusely adherent Escherichia coli as a cause of acute diarrhea in young children in Northeast Brazil: a case-control study. *J Clin Microbiol* **40**, 645-648.

Schmidt, C., Giese, T., Ludwig, B., Mueller-Molaian, I., Marth, T., Zeuzem, S., Meuer, S. C. & Stallmach, A. (2005). Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm Bowel Dis* **11**, 16-23.

Sears, C. L. & Kaper, J. B. (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev* **60**, 167-215.

Seaver, L. C. & Imlay, J. A. (2001a). Hydrogen peroxide fluxes and compartmentalization inside growing Escherichia coli. *J Bacteriol* **183**, 7182-7189.

Seaver, L. C. & Imlay, J. A. (2001b). Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli. *J Bacteriol* **183**, 7173-7181.

Seaver, L. C. & Imlay, J. A. (2004). Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *J Biol Chem* **279**, 48742-48750.

Seksik P, S. H., Lepage P, Vasquez N, Manichanh C, Mangin I, Pochart P, Dore J, Marteau P. (2006). Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. . *Aliment Pharmacol Ther* **24**, 11- 18.

Selander, R. K. & Levin, B. R. (1980). Genetic diversity and structure in Escherichia coli populations. *Science* **210**, 545-547.

Semjen, D., Fabos, Z., Pakodi, F., Vincze, A., Szabo, I., Degrell, P., Csete, M. & Tornoczky, T. (2011). Renal involvement in Crohn's disease: granulomatous inflammation in the form of mass lesion. *Eur J Gastroenterol Hepatol* **23**, 1267-1269.

Seo, K. S., Kim, J. W., Park, J. Y., Viall, A. K., Minnich, S. S., Rohde, H. N., Schnider, D. R., Lim, S. Y., Hong, J. B., Hinnebusch, B. J., O'Loughlin, J. L., Deobald, C. F., Bohach, G. A., Hovde, C. J. & Minnich, S. A. (2012). The role of an intimin/invasin/autotransporter-like protein (Ilp) in Yersinia pestis virulence. *Infect Immun* **10**, 3559-3569.

Sepehri, S., Kotlowski, R., Bernstein, C. N. & Krause, D. O. (2009). Phylogenetic analysis of inflammatory bowel disease associated Escherichia coli and the fimH virulence determinant. *Inflamm Bowel Dis* **15**, 1737-1745.

Servin, A. L. (2005). Pathogenesis of Afa/Dr diffusely adhering Escherichia coli. *Clin Microbiol Rev* **18**, 264-292.

Shimada, K., Crother, T. R., Karlin, J., Chen, S., Chiba, N., Ramanujan, V. K., Vergnes, L., Ojcius, D. M. & Arditi, M. (2011). Caspase-1 dependent IL-1beta secretion is critical for host defense in a mouse model of Chlamydia pneumoniae lung infection. *PLoS ONE* **6**, e21477.

Silhavy, T. J., Berman, D. M. & Enquist, L. W. (1984). Preparation of a P1Tn9clr100 lysate. *In Experiments with Gene Fusions*.

Simon, A. & Biot, E. (2010). ANAIS: analysis of NimbleGen arrays interface. *Bioinformatics* **26**, 2468-2469.

Simonsen, K. T., Nielsen, G., Bjerrum, J. V., Kruse, T., Kallipolitis, B. H. & Moller-Jensen, J. (2011). A role for the RNA chaperone Hfq in controlling adherent-invasive Escherichia coli colonization and virulence. *PLoS ONE* **6**, e16387.

Simpson, K. W., Dogan, B., Rishniw, M., Goldstein, R. E., Klaessig, S., McDonough, P. L., German, A. J., Yates, R. M., Russell, D. G., Johnson, S. E., Berg, D. E., Harel, J., Bruant, G., McDonough, S. P. & Schukken, Y. H. (2006). Adherent and invasive Escherichia coli is associated with granulomatous colitis in boxer dogs. *Infect Immun* **74**, 4778-4792.

Singh, S. B., Davis, A. S., Taylor, G. A. & Deretic, V. (2006). Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* **313**, 1438-1441.

Smith, M. S. & Wakefield, A. J. (1993). Viral association with Crohn's disease. *Ann Med* **25**, 557-561.

Smith, S. G. & Dorman, C. J. (1999). Functional analysis of the FimE integrase of Escherichia coli K-12: isolation of mutant derivatives with altered DNA inversion preferences. *Mol Microbiol* **34**, 965-979.

Snyder, W. B., Davis, L. J., Danese, P. N., Cosma, C. L. & Silhavy, T. J. (1995). Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. *J Bacteriol* **177**, 4216-4223.

Sohel, I., Puente, J. L., Ramer, S. W., Bieber, D., Wu, C. Y. & Schoolnik, G. K. (1996). Enteropathogenic Escherichia coli: identification of a gene cluster coding for bundle-forming pilus morphogenesis. *J Bacteriol* **178**, 2613-2628.

Sokurenko, E. V., Chesnokova, V., Dykhuizen, D. E., Ofek, I., Wu, X. R., Krogfelt, K. A., Struve, C., Schembri, M. A. & Hasty, D. L. (1998). Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. *Proc Natl Acad Sci U S A* **95**, 8922-8926.

Somerville, K. W., Logan, R. F., Edmond, M. & Langman, M. J. (1984). Smoking and Crohn's disease. *Br Med J (Clin Res Ed)* **289**, 954-956.

Souza, V., Rocha, M., Valera, A. & Eguiarte, L. E. (1999). Genetic structure of natural populations of Escherichia coli in wild hosts on different continents. *Appl Environ Microbiol* **65**, 3373-3385.

Spinelli, A., Correale, C., Szabo, H. & Montorsi, M. (2009). Intestinal fibrosis in Crohn's disease: medical treatment or surgery? *Curr Drug Targets* **11**, 242-248.

Splettstoesser, W. D. & Schuff-Werner, P. (2002). Oxidative stress in phagocytes--"the enemy within". *Microsc Res Tech* **57**, 441-455.

Stanley, T. G. & Wilson, I. (2003). Multilocus enzyme electrophoresis: a practical guide. *Mol Biotechnol* **24**, 203-220.

Steele-Mortimer, O., St-Louis, M., Olivier, M. & Finlay, B. B. (2000). Vacuole acidification is not required for survival of Salmonella enterica serovar typhimurium within cultured macrophages and epithelial cells. *Infect Immun* **68**, 5401-5404.

Steinberg, K. M. & Levin, B. R. (2007). Grazing protozoa and the evolution of the Escherichia coli O157:H7 Shiga toxin-encoding prophage. *Proc Biol Sci* **274**, 1921-1929.

Stentebjerg-Olesen, B., Chakraborty, T. & Klemm, P. (1999). Type 1 fimbriation and phase switching in a natural Escherichia coli fimB null strain, Nissle 1917. *J Bacteriol* **181**, 7470-7478.

Stevens, J. M., Galyov, E. E. & Stevens, M. P. (2006). Actin-dependent movement of bacterial pathogens. *Nat Rev Microbiol* **4**, 91-101.

Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000). Two-component signal transduction. *Annu Rev Biochem* **69**, 183-215.

Stohl, E. A. & Seifert, H. S. (2006). Neisseria gonorrhoeae DNA recombination and repair enzymes protect against oxidative damage caused by hydrogen peroxide. *J Bacteriol* **188**, 7645-7651.

Storz, G. & Imlay, J. A. (1999). Oxidative stress. *Curr Opin Microbiol* **2**, 188-194.

Strober, W., Fuss, I. J. & Blumberg, R. S. (2002). The immunology of mucosal models of inflammation. *Annu Rev Immunol* **20**, 495-549.

Strober, W., Fuss, I. & Mannon, P. (2007). The fundamental basis of inflammatory bowel disease. *J Clin Invest* **117**, 514-521.

Strober, W. (2011). Adherent-invasive E. coli in Crohn disease: bacterial "agent provocateur". *J Clin Invest* **121**, 841-844.

Suau, A., Bonnet, R., Sutren, M., Godon, J. J., Gibson, G. R., Collins, M. D. & Dore, J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* **65**, 4799-4807.

Subramanian, S., Roberts, C.L., Anthony Hart, C., Martin, H., M., Edwards, S.W., Rhodes, J. and Campbell, B.J. (2008). Replication of Colonic Crohn's Disease Mucosal Escherichia coli Isolates within Macrophages and Their Susceptibility to Antibiotics. *AAC* **52**, 427-434.

Sugihara, S., Orikasa, Y. & Okuyama, H. The Escherichia coli highly expressed entD gene complements the pfaE deficiency in a pfa gene clone responsible for the biosynthesis of long-chain n-3 polyunsaturated fatty acids. *FEMS Microbiol Lett* **307**, 207-211.

Suvarnapunya, A. E., Lagasse, H. A. & Stein, M. A. (2003). The role of DNA base excision repair in the pathogenesis of Salmonella enterica serovar Typhimurium. *Mol Microbiol* **48**, 549-559.

Suvarnapunya, A. E. & Stein, M. A. (2005). DNA base excision repair potentiates the protective effect of Salmonella Pathogenicity Island 2 within macrophages. *Microbiology* **151**, 557-567.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M. & Lochs, H. (2002). Mucosal flora in inflammatory bowel disease. *Gastroenterology* **122**, 44-54.

Szurmant, H., White, R. A. & Hoch, J. A. (2007). Sensor complexes regulating two-component signal transduction. *Curr Opin Struct Biol* **17**, 706-715.

Thom, S., Warhurst, D. & Drasar, B. S. (1992). Association of Vibrio cholerae with fresh water amoebae. *J Med Microbiol* **36**, 303-306.

Thompkins, K., Chattopadhyay, B., Xiao, Y., Henk, M. C. & Doerrler, W. T. (2008). Temperature sensitivity and cell division defects in an Escherichia coli strain with mutations in yghB and yqjA, encoding related and conserved inner membrane proteins. *J Bacteriol* **190**, 4489-4500.

Timmer, A., Sutherland, L. R. & Martin, F. (1998). Oral contraceptive use and smoking are risk factors for relapse in Crohn's disease. The Canadian Mesalamine for Remission of Crohn's Disease Study Group. *Gastroenterology* **114**, 1143-1150.

Tobin, M. V., Logan, R. F., Langman, M. J., McConnell, R. B. & Gilmore, I. T. (1987). Cigarette smoking and inflammatory bowel disease. *Gastroenterology* **93**, 316-321.

Torok, H. P., Glas, J., Endres, I., Tonenchi, L., Teshome, M. Y., Wetzke, M., Klein, W., Lohse, P., Ochsenkuhn, T., Folwaczny, M., Goke, B., Folwaczny, C., Muller-Myhsok, B. & Brand, S. (2009). Epistasis between Toll-like receptor-9 polymorphisms and variants in NOD2 and IL23R modulates susceptibility to Crohn's disease. *Am J Gastroenterol* **104**, 1723-1733.

Traub, S., von Aulock, S., Hartung, T. & Hermann, C. (2006). MDP and other muropeptides--direct and synergistic effects on the immune system. *J Endotoxin Res* **12**, 69-85.

Troup, B., Jahn, M., Hungerer, C. & Jahn, D. (1994). Isolation of the hemF operon containing the gene for the Escherichia coli aerobic coproporphyrinogen III oxidase by in vivo complementation of a yeast HEM13 mutant. *J Bacteriol* **176**, 673-680.

Tsianos, E. V., Katsanos, K. H. & Tsianos, V. E. (2012). Role of genetics in the diagnosis and prognosis of Crohn's disease. *World J Gastroenterol* **18**, 105-118.

Tsuzuki, M., Ishige, K. & Mizuno, T. (1995). Phosphotransfer circuitry of the putative multi-signal transducer, ArcB, of Escherichia coli: in vitro studies with mutants. *Mol Microbiol* **18**, 953-962.

Turner, S. M., Chaudhuri, R. R., Jiang, Z. D., DuPont, H., Gyles, C., Penn, C. W., Pallen, M. J. & Henderson, I. R. (2006). Phylogenetic comparisons reveal multiple acquisitions of the toxin genes by enterotoxigenic Escherichia coli strains of different evolutionary lineages. *J Clin Microbiol* **44**, 4528-4536.

Uhlen, P., Laestadius, A., Jahnukainen, T. & other authors (2000). Alpha-haemolysin of uropathogenic E. coli induces Ca2+ oscillations in renal epithelial cells. *Nature* **405**, 694-697.

Vaerewijck, M. J., Huys, G., Palomino, J. C., Swings, J. & Portaels, F. (2005). Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* **29**, 911-934.

Van Houten, B., Croteau, D. L., DellaVecchia, M. J., Wang, H. & Kisker, C. (2005). 'Close-fitting sleeves': DNA damage recognition by the UvrABC nuclease system. *Mutat Res* **577**, 92-117.

Van Kruiningen, H. J., Colombel, J. F., Cartun, R. W., Whitlock, R. H., Koopmans, M., Kangro, H. O., Hoogkamp-Korstanje, J. A., Lecomte-Houcke, M., Devred, M. & Paris, J. C. (1993). An in-depth study of Crohn's disease in two French families. *Gastroenterology* **104**, 351-360.

Van Kruiningen, H. J., Ganley, L. M. & Freda, B. J. (1997). The role of Peyer's patches in the age-related incidence of Crohn's disease. *J Clin Gastroenterol* **25**, 470-475.

Van Limbergen, J., Russell, R. K., Nimmo, E. R., Ho, G. T., Arnott, I. D., Wilson, D. C. & Satsangi, J. (2007). Genetics of the innate immune response in inflammatory bowel disease. *Inflamm Bowel Dis* **13**, 338-355.

Vazeille, E., Bringer, M.-A. s., Gardarin, A. l. & other authors (2011). Role of Meprins to Protect Ileal Mucosa of Crohn's Disease Patients from Colonization by Adherent-Invasive <italic>E. coli</italic>. *PLoS ONE* **6**, e21199.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P. & Fang, F. C. (2000). Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**, 1655-1658.

Vogt, S. L., Nevesinjac, A. Z., Humphries, R. M., Donnenberg, M. S., Armstrong, G. D. & Raivio, T. L. (2010). The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic Escherichia coli bundle-forming pilus. *Mol Microbiol* **76**, 1095-1110.

Wai, S. N., Takade, A. & Amako, K. (1995). The release of outer membrane vesicles from the strains of enterotoxigenic Escherichia coli. *Microbiol Immunol* **39**, 451-456.

Walk, S. T., Alm, E. W., Gordon, D. M., Ram, J. L., Toranzos, G. A., Tiedje, J. M. & Whittam, T. S. (2009). Cryptic lineages of the genus Escherichia. *Appl Environ Microbiol* **75**, 6534-6544.

Wang, C., Mendonsa, G. R., Symington, J. W., Zhang, Q., Cadwell, K., Virgin, H. W. & Mysorekar, I. U. (2012). Atg16L1 deficiency confers protection from uropathogenic Escherichia coli infection in vivo. *Proc Natl Acad Sci U S A* **109**, 11008-11013.

Wang, X., Preston, J. F., 3rd & Romeo, T. (2004). The pgaABCD locus of Escherichia coli promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* **186**, 2724-2734.

Watanabe, T., Okada, A., Gotoh, Y. & Utsumi, R. (2008). Inhibitors targeting two-component signal transduction. *Adv Exp Med Biol* **631**, 229-236.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., McClelland, M. & Shi, Y. (2010). The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *J Biol Chem* **286**, 5529-5539.

Weiser, J. A., Markowitz, D. M., Husain, S. & Grossman, M. E. (2011). An unusual cutaneous manifestation of Crohn's disease. *Skinmed* **9**, 196-198.

Werner, T., Wagner, S. J., Martinez, I., Walter, J., Chang, J. S., Clavel, T., Kisling, S., Schuemann, K. & Haller, D. (2010). Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. *Gut* **60**, 325-333.

Whitfield, N. N., Byrne, B. G. & Swanson, M. S. (2009). Mouse macrophages are permissive to motile Legionella species that fail to trigger pyroptosis. *Infect Immun* **78**, 423-432.

Whittam, T. S. (1989). Clonal dynamics of Escherichia coli in its natural habitat. *Antonie Van Leeuwenhoek* **55**, 23-32.

Whittam, T. S., Wolfe, M. L., Wachsmuth, I. K., Orskov, F., Orskov, I. & Wilson, R. A. (1993). Clonal relationships among Escherichia coli strains that cause hemorrhagic colitis and infantile diarrhea. *Infect Immun* **61**, 1619-1629.

Wildschutte, H., Wolfe, D. M., Tamewitz, A. & Lawrence, J. G. (2004). Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella. *Proc Natl Acad Sci U S A* **101**, 10644-10649.

Wildschutte, H. & Lawrence, J. G. (2007). Differential Salmonella survival against communities of intestinal amoebae. *Microbiology* **153**, 1781-1789.

Wine, E., Ossa, J. C., Gray-Owen, S. D. & Sherman, P. M. (2011). Adherent-invasive Escherichia coli target the epithelial barrier. *Gut Microbes* **1**, 80-84.

Winiecka-Krusnell, J., Wreiber, K., von Euler, A., Engstrand, L. & Linder, E. (2002). Free-living amoebae promote growth and survival of Helicobacter pylori. *Scand J Infect Dis* **34**, 253-256.

Winkler, J. & Kao, K. C. (2012). Harnessing recombination to speed adaptive evolution in Escherichia coli. *Metab Eng* **5**, 487-495.

Winterbourn, C. C. & Kettle, A. J. (2012). Redox Reactions and Microbial Killing in the Neutrophil Phagosome. *Antioxid Redox Signal* (Epub ahead of print).

Wirth, T., Falush, D., Lan, R. & other authors (2006). Sex and virulence in Escherichia coli: an evolutionary perspective. *Mol Microbiol* **60**, 1136-1151.

Wolf, M. K. (1997). Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic Escherichia coli. *Clin Microbiol Rev* **10**, 569-584.

Xavier, R. J. & Podolsky, D. K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427-434.

Xiong, Y., Wang, P., Lan, R. & other authors (2012). A Novel Escherichia coli O157:H7 Clone Causing a Major Hemolytic Uremic Syndrome Outbreak in China. *PLoS ONE* **7**, e36144.

Xu, Z., Dziarski, R., Wang, Q., Swartz, K., Sakamoto, K. M. & Gupta, D. (2001). Bacterial peptidoglycan-induced tnf-alpha transcription is mediated through the transcription factors Egr-1, Elk-1, and NF-kappaB. *J Immunol* **167**, 6975-6982.

Yamamoto, K. & Ishihama, A. (2005). Transcriptional response of Escherichia coli to external copper. *Mol Microbiol* **56**, 215-227.

Yamamoto, K. & Ishihama, A. (2006). Characterization of copper-inducible promoters regulated by CpxA/CpxR in Escherichia coli. *Biosci Biotechnol Biochem* **70**, 1688-1695.

Yamamoto, T., Kaneko, M., Changchawalit, S., Serichantalergs, O., Ijuin, S. & Echeverria, P. (1994). Actin accumulation associated with clustered and localized adherence in Escherichia coli isolated from patients with diarrhea. *Infect Immun* **62**, 2917-2929.

Yamazaki, K., McGovern, D., Ragoussis, J. & other authors (2005). Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. *Hum Mol Genet* **14**, 3499-3506.

Yoong, P., Cywes-Bentley, C. & Pier, G. B. (2012). Poly-N-Acetylglucosamine Expression by Wild-Type Yersinia pestis Is Maximal at Mammalian, Not Flea, Temperatures. *MBio* **3**.

Yu, J., Oragui, E. E., Stephens, A., Kroll, J. S. & Venkatesan, M. M. (2001). Inactivation of DsbA alters the behaviour of Shigella flexneri towards murine and human-derived macrophage-like cells. *FEMS Microbiology Letters* **204**, 81-88.

Zare, H., Sangurdekar, D., Srivastava, P., Kaveh, M. & Khodursky, A. (2009). Reconstruction of Escherichia coli transcriptional regulatory networks via regulon-based associations. *BMC Syst Biol* **3**, 39.

Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M. G. & Alon, U. (2006). A comprehensive library of fluorescent transcriptional reporters for Escherichia coli. *Nat Methods* **3**, 623-628.

Zavros, Y. & Rogler, G. (2012). Variants in Autophagy Genes Trigger Susceptibility to Both Crohn's Disease and Helicobacter pylori Infection. *Gastroenterology*.

Zhao, Y., Wang, D., Nakka, S., Sundin, G. W. & Korban, S. S. (2009). Systems level analysis of two-component signal transduction systems in Erwinia amylovora: role in virulence, regulation of amylovoran biosynthesis and swarming motility. *BMC Genomics* **10**, 245.

Zheng, M., Aslund, F. & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**, 1718-1721.

Zychlinsky, A., Kenny, B., Menard, R., Prevost, M. C., Holland, I. B. & Sansonetti, P. J. (1994). IpaB mediates macrophage apoptosis induced by Shigella flexneri. *Mol Microbiol* **11**, 619-627.